Revitalising the rudimentary replacement dentition in the mouse

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SUMMARY

Most mammals have two sets of teeth (diphyodont), a deciduous dentition replaced by a permanent dentition, however the mouse possesses only one tooth generation (monophyodont). In diphyodonts the replacement tooth forms on the lingual side of the first tooth from the successional dental lamina. This lamina expresses the stem/progenitor marker Sox2 and has activated Wnt/B-catenin signalling at its tip. Although the mouse does not replace its teeth a transient rudimentary successional dental lamina (RSDL) still forms during development. The mouse RSDL houses Sox2-positive cells, but no Wnt/B-catenin signalling. Here we show that stabilizing Wnt/B-catenin signaling in the RSDL in the mouse leads to proliferation of the RSDL and formation of lingually positioned teeth. Although Sox2 has been shown to repress Wnt activity, overexpression of Wnts leads to a downregulation of Sox2, suggesting a negative feedback loop in the tooth. In the mouse the first tooth represses the formation of the replacement, and isolation of the RSDL is sufficient to induce formation of a new tooth germ. Our data highlights key mechanisms that may have influenced the evolution of replacement teeth.

Introduction

Considerable effort has been made in the past decade using a rich variety of animal models to understand the molecular mechanisms underlying tooth replacement (Handrigan et al., 2010; Wu et al., 2013; Jussila et al., 2014; Yamanaka et al., 2007; Järvinen et al., 2008; Gaete and Tucker, 2013; Fraser et al., 2006). Across the animal kingdom, tooth development is a conserved and tightly regulated process, involving two-way molecular communication between an initially inductive epithelium and neural crest-derived mesenchyme. Together these two tissues form the tooth germ, passing through well-described developmental stages (bud, cap, bell) before starting to produce mineralized tissue and finally erupt and become functional (Luckett, 1993). As teeth are worn down throughout life, new replacements are often required. Whilst certain animals possess the ability of continuously replenishing their lost dentitions throughout their lifetime (Gaete and Tucker, 2013; Rasch et al., 2016; Handrigan and Richman, 2010b), in many others replacement is either limited to two generations or does not occur at all. This restriction to two generations, as observed in most mammals, is linked to the evolution of more complex tooth shapes adapted for certain diets, different tooth types and precise occlusion. There, therefore, appears a trade-off between tooth replacement number and complexity (Javinen et al., 2006)(Tucker and Fraser, 2014).

In mammals replacement teeth form from the dental lamina on the lingual side of the first tooth, known as the successional lamina (Järvinen et al., 2009; Juuri et al., 2013; Olley et al., 2014; Ooe, 1891; Berkovitz, 1972; van Nievelt and Smith, 2005; Wang et al., 2007). Later in embryonic development, and after the first generation of teeth have commenced development, the dental lamina begins to regress via a combination of processes including cell death, disruption of the basement membrane and epithelial-to-mesenchymal transformation, preventing further tooth development (Buchtová et al., 2012). Sox2, a marker of stem/progenitor cells, localises to the dental lamina in diphyodont and polyphyodont animals (Juuri et al., 2013; Gaete and Tucker, 2013) and Sox2+ cells in the dental placode give rise to all epithelial lineages of the incisor and

molar teeth (Juuri et al., 2012, 2013). In the successional lamina Sox2 expression is excluded from the very tip of the lamina, a region that expresses high levels of Wnt signalling, as shown by expression of the Wnt effector Lef1 (Juuri et al., 2013; Handrigan and Richman, 2010a; Gaete and Tucker, 2013) and localisation of nuclear β-catenin (Jussila et al., 2014). This complementary pattern of expression of Sox2 and activation of the Wnt pathway in the successional lamina is conserved in both diphyodont mammals and reptiles and therefore appears an essential component of tooth regeneration.

In taste buds and lung, Sox2 has been shown to antagonise Wnt signalling (Okubo et al., 2006; Hashimoto et al., 2012) with Sox2 capable of directly binding to the β-catenin/TCF/LEF1 transcriptional complex (Mansukhani et al., 2005). The mutually exclusive expression of Sox2 and canonical Wnt signalling in the successional lamina therefore suggests that Sox2 may also inhibit canonical Wnt signalling in this part of the tooth, confining Wnt activity to the cells at the tip of the lamina.

Whilst most mammals have two sets of teeth, a deciduous dentition replaced by a permanent dentition, the mouse possesses only one tooth generation. In order to cope with tooth wear the incisors of the mouse are continuously growing (hypselodonty) with epithelial and mesenchymal stem cell niches housed within and around the labial cervical loop to provide a constant ameloblast and odontoblast progenitors (Li et al., 2012; Biehs et al., 2013; Kaukua et al., 2014; Juuri et al., 2012). The labial cervical loop expresses Sox2, with canonical Wnt signalling excluded from this region (Juuri et al., 2012; Sun et al., 2016a). The mouse molars are not continuously growing and form from a single placode in an anterior-to-posterior direction. Molars of mammals are not replaced, this ability having been lost more than 200 million years ago (Kielan-Jaworowska et al., 2004). Despite this, a transient epithelial rudiment known as the rudimentary successional dental lamina (RSDL) is observed in many mammals during molar development (Dosedělová et al., 2015). The mouse RSDL protrudes from the lingual side of molar tooth germs, in a region equivalent to the lamina that forms successional teeth in diphyodont mammals, and houses Sox2-positive cells

(Dosedělová et al., 2015). The RSDL shows high proliferation during early stages of formation (E16.5-E18.5), however after this point proliferation is reduced and the lamina regresses (Dosedělová et al., 2015).

Why this RSDL regresses and fails to make a second tooth germ is an interesting question, given the initial formation of a Sox2 positive structure. One possibility is that Sox2 inhibits Wnt activity in the tip of the forming successional lamina, preventing formation of a second tooth. Wnt activity has been shown to be absent, for example in the regressing successional dental lamina of bearded dragon teeth, which are not replaced (Handrigan and Richman, 2010b).

In the alligator, nuclear β-catenin expressed in the dental lamina during the initiation phase has been linked with an involvement of the Wnt pathway in regulating the quiescent and proliferative states of dental tissues during tooth regeneration (Wu et al., 2013), while in the leopard gecko dental epithelium Wnt signalling was suggested to regulate stem cell fate by inducing slow-cycling stem cells to proliferate (Handrigan et al., 2010).
In mice there have been a number of studies where activation of canonical Wnt signalling via different transgenic lines in the oral epithelium has led to the production of a large number of supernumerary teeth (Järvinen et al., 2006; Xavier et al., 2015; Wang et al., 2009). In snakes a similar general overexpression of Wnt signalling also leads to the formation of unregulated tooth production in explant culture (Gaete et al., 2013). In the mouse overexpression of Wnt/β-catenin in the mesenchyme has recently been shown to inhibit tooth number, with over expression of Wnts in the mesenchyme counteracting the effects of overexpression in the epithelium (Jarvinen et al., 2018). Precise regulation of Wnt signalling is therefore essential for the correct control of the number of replacement tooth generations.

Here we have investigated what prevents replacement tooth formation in the mouse and try to re-waken the tooth programme in the RSDL. For this we compared progenitor marker expression in the dental tissues of the minipig, a mammal with two sets of teeth, with those of the monophyodont mouse, to

pinpoint similarities and differences that might be responsible for cessation of tooth replacement. Based on species-specific differences in progenitor cell localization, we then tested whether activation of signalling in the rudimentary dental lamina could revitalize tooth replacement in the mouse. This data could lead to a better understanding of the molecular mechanisms required for tooth regeneration, with important practical implications for the field.

Materials and Methods

Minipig tissue

Strain LiM minipig embryos were collected at the Libechov animal facility in the Czech Republic (ethical approval 020/2010). Insemination day is regarded as day 0 of gestation. Embryos were collected at embryonic day (E) 67 and fixed in 4% paraformaldehyde overnight. Samples were washed in phosphate buffered saline, dehydrated through a series of increasing methanol concentrations, followed by isopropanol. Heads were embedded in paraffin wax after clearing in 1,2,3,4-tetrahydronaphtalene. Samples were sectioned frontally at a thickness of 5 micrometres and collected for fluorescence immunohistochemical staining. Sections showing the successional lamina were taken at the level of the fourth premolar, while the interdental lamina sections were anterior to the fourth premolar.

Mouse strains and collection

Sox2^{CreERT2} mice were generated and described by Andoniadou et al. (2013). The *Ctnnb1-lox(ex3)* mice have been previously described by Harada et al. (1999). The two strains were mated to generate *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)}$* embryos. Mice were also mated to Rosa-tdTomato (Madisen et al., 2010) heterozygous mice to generate *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)/+}$;*R26$^{TOM/+}$ mice in order to allow overexpressing cells to be followed. Axin2/LacZ were used as previously described as a readout of canonical Wnt activity (Lohi et al., 2010).

Mating of C57Bl6 adult mice was set up in the evening, with vaginal plug checks performed in the morning. Embryonic day (E) 0.5 was marked at midday of the day a vaginal plug was observed. Adult pregnant females were injected intraperitoneally with 20mg/ml Tamoxifen and 10mg/Progesterone dissolved in 10% ethanol in corn oil at E15.5 and E16.0 and culled by exposure to CO_2 gas at E17.5 (for Kidney capsule and explant culture experiments) or E18.5 (for wax embedding). Controls were injected with corn oil only. A single Bromodeoxyuridine (BrdU) injection was given to pregnant mice 30 minutes before culling.

Embryos were dissected out, genotyped and processed for wax embedding. For histology studies, eight micrometre-thick paraffin sections were stained with Picro Sirius Red, Haematoxylin and Alcian Blue (trichrome stain).

N= 8 *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)}$* and n= 8 control (Tamoxifen injected *Sox2$^{CreERT2/-}$;Ctnnb1$^{lox(ex3)}$*) or (Corn oil injected *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)}$*) embryos from three separate litters were used for this study. For the 6-day culture experiment, n=3 control and n=5 *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)}$* samples were used. For the kidney capsule experiments, n=2 control and n=3 *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)}$* samples were used. For the BIO culture experiments, n=3 control and n=4 treated samples were assessed for whole mount immunohistochemistry.

All culling followed schedule one methods as approved by the UK Home Office and was performed by trained individuals. All procedures were performed under approved personal and project licences in accordance with the Animal (Scientific Procedures) Act of 1986, United Kingdom.

Immunohistochemistry

Paraffin sections were dewaxed in xylene and rehydrated through 100%, 90% and 70% methanol, followed by a wash in deionised water. Heat-mediated antigen retrieval was performed in a solution of tris-EDTA, pH 9.0 (DAKO) for 15

minutes, followed by cooling and incubation with blocking solution (10% goat serum, 0.1% Triton in PBS). Sections were incubated at 4 degrees Celsius overnight with primary antibodies (rabbit anti-Sox2 2748s, rabbit anti-Sox9 AB5535, rabbit anti-Beta catenin C2206, rabbit anti-laminin L9393, rat anti-BrdU AB6326) at a concentration of 1:200 in blocking solution. Incubation with the secondary antibody (Alexa 568 goat anti-rabbit A11034, or goat anti-rat A11077) was carried out the next day over a period of two hours at room temperature. All antibodies used showed expression that agreed with published expression patterns in other species, or in other tissues in the head. Imaging was carried out using a Leica SP5 confocal microscope. Images were cropped and adjusted for levels using Adobe Photoshop.

Whole mount and paraffin section *in situ* hybridization

Mouse probes for *Shh* (EcoR1, T7)*, Fgf3* (HindIII, T7)*, Fgf4* (XbaI)*, Bmp4* (EcoR1, Sp6)*, Pax9* (BamHI, T3)*, Ectodin* (NotI, T7) were linearised and transcribed. In situ hybridisation of craniofacial sections was carried out as previously described in Gaete et al., 2015.

3D reconstruction

Serial histological sections containing the upper molar tooth set of E18.5 control and *Sox2$^{CreERT2/+}$;Ctnnb1$^{lox(ex3)/+}$* embryos were imaged in an anterior-to-posterior sequence. The images were imported in FIJI (FIJI is Just Image J 1.47v), grouped as a stack and aligned using a combination of automatic and manual alignments. The dental tissues were painted manually over each image and from this a 3D object was generated, which was then photographed from labial, lingual and oral perspectives.

Kidney capsule surgery

Sox2CreGOFTOM pregnant females were injected with tamoxifen and progesterone at 15.5 days of gestation and embryos were collected at embryonic day 17.5. Control and *Sox2*$^{CreERT2/+;}$*Ctnnb1*$^{lox(ex3)}$ tooth germs were carefully dissected out in DMEM/F12 supplemented with penicillin and streptomycin. Three 7-week-old CD1 males were anaesthetised using ketamine 80mg/kg and xylazine 16mg/kg by intraperitoneal injection. The area of incision on the dorsal right side of the mouse was cleaned with 70% ethanol, then a 1 cm long incision was made above the right kidney area. The kidney was gently placed outside of the abdominal cavity and two dental organ explants were inserted under the membrane covering the kidney. The kidneys were then placed back inside the abdominal cavity, and the incision was sutured. Two weeks after the surgery, the mice were sacrificed in a CO_2 chamber and the kidneys were removed, fixed in 4% paraformaldehyde and scanned using a GE locus SP microCT scanner. Images were reconstructed using microview software. After scanning samples were decalcified and prepared for histological analysis.

Whole explant culture

Lower jaw molar regions were dissected at E14.5 and incubated with Wnt activator BIO for two days using a modified Trowell culture method. The BIO was dissolved in DMSO and added to Advanced Dulbecco's Modified Eagle Medium F12 (DMEM F12) culture medium (plus Glutamax and Pen/Strep) at a final concentration of 20uM. Control cultures were incubated with DMSO. After two days samples were processed for whole mount immunofluorescence.

Isolation of the RSDL

Lower jaws were dissected at E16.5 and E17.5 from WT and Axin2LacZ/+ embryos. The molar placodes were isolated and the bone removed using fine tungsten needles. Placodes were then sliced into 250um frontal sections using a McIlwean tissue chopper. First molar slices with clear RSDL on the lingual side were selected. Slices where the RSDL was not clear were excluded. In some cases the slices were cultured for several hours before cutting to allow more accurate

visualisation of the RSDL. The RSDL was then separated from the rest of the developing tooth using a scapel blade in half the slices (N = 10 for each stage). Slices were photographed before and after isolation using a Leica Microscope and cultured on filters for 4 days (Alfaqeeh et al., 2013). After culture the slices were fixed for 15 minutes in 4% PFA. Axin2lacZ/+ positive slices were stained with X-galactosidase for 6 hours at 37 degrees C. Slices were then permeablised in 0.1% PBT for several hours, and then place in DAPI to stain the nuclei. Slices were mounted and photographed on a Leica SP5 confocal microscope.

Results

Similarities between the mouse RSDL and non-tooth producing regions of the dental lamina in the minipig

In diphyodonts, such as the minipig, the forming tooth germs are connected by a dental lamina, a continuous epithelial sheet spanning the length of the jaw. The dental lamina between the tooth germs is called the interdental lamina. The interdental lamina does not produce teeth, in contrast to the tooth producing successional dental lamina located adjacent to the first generation teeth. Sox2 and Sox9 have recently been shown to have complementary patterns of expression in the growing molar tail, which will form the serially added molars at the back of the mouth (Gaete et al., 2015). We therefore analysed the expression of these two progenitor markers in the two parts of the minipig dental lamina, and compared their expression to the RSDL in the mouse.

In the minipig, Sox2 was localised on the lingual side of the successional lamina of the 4th premolar, and was markedly absent from the apical tip and the labial end of the structure, agreeing with expression shown in other diphyodonts (Juuri et al., 2013) (Figure 1 A). In contrast, in the interdental lamina, just anterior to the 4th premolar, Sox2 was present all the way to the apex (Figure 1 B). Sox9 was expressed in a complementary pattern to that of Sox2, with fewer positive cells

in the lingual part of the deep dental lamina and abundant positive cells in the middle epithelium (Figure 1 E). Sox9, like Sox2, however, was excluded from the labial and lingual epithelium at the tip of the successional dental lamina (Figure 1 E). In the interdental lamina Sox9 was expressed in the lingual epithelium all the way to the tip (Figure 1 F). The lingual and labial epithelium at the tip of the successional lamina housed mostly Wnt-active cells, shown by nuclear localisation of β-catenin in both the epithelium and surrounding mesenchyme when viewed at high power (Figure 1 I). The interdental lamina, in stark contrast, was completely devoid of cells with nuclear β-catenin, indicating absence of canonical Wnt signalling in this structure (Figure 1 J). This key difference could be the determining factor as to whether the dental lamina is poised to give rise to a further generation of teeth or not, with extended Sox2 and Sox9 expression potentially inhibiting Wnt activity in the interdental region to prevent further tooth formation.

In the mouse RSDL at E18.5, Sox2 was present in the RSDL and lingual oral epithelium but appeared absent from the tip. At this stage therefore the RSDL has some characteristics of the minipig successional lamina (Figure 1 C), however, unlike the minipig the tip of the RSDL expressed high levels of Sox9 and no nuclear β-catenin (Figure 1 G, K). As the RSDL started to regress at P0, Sox2 and Sox9 became co-localised in the tip of the rudiment, similar to the situation observed in the minipig interdental lamina. Again at this stage the mouse RSDL had no nuclear β-catenin. This data clearly suggests that it is lack of Wnt/β-catenin signalling in the RSDL that prevents the formation of a second generation of teeth, agreeing with previous analysis of rudimentary laminas in monophyodont reptiles (Richman & Handrigan, 2011).

The mouse RSDL has the potential to make replacement tooth germs

As Sox2 can inhibit activation of canonical Wnt signalling in other systems we wanted to bypass the potential inhibition of Wnt activity in the mouse RSDL

using the cre-lox system to stabilise Wnt/β-catenin signalling in the RSDL. To do this we constitutively activated Wnt/β-catenin signalling in Sox2 positive cells (Sox2ert2cre) and injected tamoxifen into pregnant mothers at E15.5 and E16.0. At this stage Sox2 has a relatively restricted expression pattern in the tooth, with positive cells in the RDSL, lingual side of the dental stalk and in a few cells of the inner enamel epithelium, in addition to more widespread expression in the oral epithelium and neighbouring vestibular lamina (Dosedělová et al., 2015; Sun et al., 2016). Embryos were collected three days later at E18.5.

Activation of Wnt signalling in the RSDL from E16.0 resulted in the formation of an enlarged dental lamina on the lingual side of the first molar with heightened proliferation, as shown by BrDU incorporation, in the epithelium and surrounding dental mesenchyme (Fig. 2 A, B). More proliferating cells were also observed on the lingual side of the second molar in the mutant compared to littermate controls (Supplementary Figure 1 A, E). Laminin immunostaining was used to outline the forming RSDL, emphasising the larger size of the RSDL after activation of Wnt signalling (Fig. 2C,D). Laminin also highlighted the pattern of blood vessels in the mesenchyme, which formed concentric whirls around the developing *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* RSDL (Fig.2 C, D).

A slightly earlier tamoxifen injection at E15.5 led to the formation of a more prominent structure forming from the free end of the dental lamina in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* embryos (Figure 3). These embryos exhibited a general thickening of the oral and palate epithelium, with multiple small epithelial protrusions throughout (figure 3 A, E). The first molars, although largely normal, had a thickened dental lamina and slightly disorganised layer of preameloblasts (Figure 3 B, F, D, H). The odontoblast layer also appeared less organised, perhaps reflecting a defect in signalling from the ameloblasts to the odontoblasts (Thesleff et al., 2001). At this stage the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* RSDL had formed a small cap-stage tooth germ, with a columnar inner enamel epithelium and condensed mesenchyme underneath it (Figure 3 C, G). The second molar showed a slightly more severe phenotype, with larger epithelial protrusions developing on its lingual side (Supplementary Figure 1 D, H). This is

consistent with the fact that the second molar is developmentally two days behind the first molar, and the expression of Sox2 is more widespread in the tooth at this earlier developmental stage.

The mice were mated to incorporate the floxed Tomato gene at the Rosa26 locus, which allowed the contribution of the cells with stabilised β-catenin to be followed. At E18.5 very few Tomato-positive cells could be observed in the developing tooth itself, as would be expected given the restricted dental expression of Sox2 at the time of tamoxifen injection (Figure 3 I). The red fluorescent cells were confined to the dental stalk and the RSDL, where a new tooth germ had developed from Sox2 lineage cells. As expected given the expression pattern of Sox2, nuclear localisation of β-catenin was observed in the $Sox2^{CreERT2/+;}Ctnnb1^{lox(ex3)}$ embryos throughout the oral epithelium, vestibular lamina and in the putative tooth germ formed in the RSDL area (Figure 3 J and Supplementary Figure 1 B, C, F, G). Interestingly, the cells with high levels of nuclear β-catenin aggregated into clusters surrounded by epithelial cells that did not have nuclear β-catenin. Positive clusters of cells were also found in the inner enamel epithelium of the second molar (supplementary Figure 1F). Given the reciprocal relationship between Sox2/9 expression and Wnt signalling in the tip of the successional lamina in the minipig (Figure 1), we investigated the expression of Sox9 after overexpression of Wnt signalling. In the controls Sox9 was expressed at the tip of the RSDL (Fig. 1 G, 3 K), while in the $Sox2^{CreERT2/+;}Ctnnb1^{lox(ex3)}$ embryos Sox9 was expressed in the forming successional tooth (Fig. 3 L), in a similar pattern to that previously described for Sox9 at the cap stage (Kawasaki et al., 2015).

Supernumerary cap stage tooth germs express enamel knot markers

To confirm the dental identity of these epithelial structures we performed *in situ* hybridisation for genes known to be pivotal for the development of tooth germs, starting with enamel knot markers. The enamel knot is an important signalling centre, which first appears within the inner enamel epithelium of the late bud tooth germ. It simulates proliferation in surrounding cells and is important for

the cuspal organisation of the tooth (Järvinen et al., 2006; Jernvall et al., 2012; Pispa et al., 2004; Thesleff et al., 2001; Jernvall et al., 1994)Tucker et al. 2000). *Sonic hedgehog* labels the cap stage primary enamel knot and is subsequently expressed in the cells of the inner enamel epithelium and preodontoblasts (Vaahtokari et al., 1996). *Shh* was absent in the RSDL of the control tooth and expressed in the preodontoblast region of the first molar (M1) (Fig. 4A). In the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* samples, *Shh* was expressed at the centre of the cap-like epithelial growth on the lingual side of M1, marking a putative enamel knot (Fig. 4 A, F). *Fgf4,* another enamel knot marker (Kettunen et al., 1998), was also expressed in the same region (Figure 4 B, G). *Fgf3* normally marks the enamel knot epithelium and underlying mesenchyme at the cap stage (Kettunen et al., 2000). *Fgf3* was absent in the RSDL area in the control situation, whereas in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* samples it was present in the enamel knot like structure and adjacent condensing mesenchyme (Figure 4 C, H). *Bmp4* is normally expressed in the enamel knot and condensing mesenchyme at the cap stage (Åberg et al., 1997; Laurikkala et al., 2003). *Bmp4* was expressed in the epithelium of the putative ectopic tooth germ, but interestingly was not expressed in the underlying mesenchyme (Figure 4 D, I). *Ectodin* (also known as *Sostdc1*) is a Wnt and Bmp inhibitor that is expressed around developing teeth but excluded from the enamel knot. In addition, in the ferret Ectodin has been shown to be expressed at the boundary between the dental lamina and the first generation tooth (Järvinen et al., 2009). *Ectodin* was dramatically upregulated in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* RSDL and in patches in the oral epithelium (Figure 4 E, J). This data suggest that the epithelial protrusions situated on the lingual side of the first molar dental lamina are cap stage supernumerary tooth germs and recapitulate normal tooth development and associated gene expression. Given the odontoblasts phenotype in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* M1 it was also evident that gene expression was disrupted in this area, with downregulation of Bmp4 and Fgf3 in the mesenchyme underlying the ameloblasts (Fig. 4 C,D,H,I).

All of the aforementioned genes were also found expressed in the protrusions on the lingual side of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* second molar (Supplementary Figure 2, A-E, G-K). *Pax9* normally marks the mesenchyme of the developing

tooth germ and was strongly expressed in the mesenchyme surrounding the epithelial protrusions on the lingual side of the second molar (Supplementary Figure 2 F, L). The vestibular lamina, which forms labial to the dental lamina and creates the lip furrow in later development, also produced epithelial cap shaped protrusions that expressed enamel knot markers (Supplementary Figure 3), confirming the odontogenic potential of this tissue.

To gain an insight into the organisation of the dental tissues affected in the *Sox2*^{CreERT2/+};*Ctnnb1*^{lox(ex3)} embryos we generated three-dimensional reconstructions from histological sections of our samples fixed at E18.5. Upon an initial inspection the *Sox2*^{CreERT2/+};*Ctnnb1*^{lox(ex3)} first and second molars appeared similar to controls, particularly when viewed from an oral or labial perspective. The phenotype was restricted to the lingual side of the dental lamina connecting the first molar to the oral epithelium, where cap-like structures could be visualised (Figure 5 A). In addition, the dental lamina connecting the first and the second molars together appeared thicker in the *Sox2*^{CreERT2/+};*Ctnnb1*^{lox(ex3)} samples compared to the controls (Figure 5 A).

Supernumerary tooth germs mineralise

To follow the development of these supernumerary teeth from the RSDL we left the mice to litter down but unfortunately all pups were eaten by the mothers. We therefore dissected out the molar dental field before birth at E17.5 and either cultured the tissue or moved to a kidney capsule for longer development. TdTom *Sox2*^{CreERT2/+};*Ctnnb1*^{lox(ex3)} were used so that the Sox2 cells and their progeny with activated B-catenin could be followed. In culture the molar tooth germs were left to develop for up to 6 days, during which time the control tooth germs exhibited normal development with expansion of the first molar and development of the second molar (Fig. 5 B, D), whereas in the *Sox2*^{CreERT2/+};*Ctnnb1*^{lox(ex3)} samples, ectopic tooth germs could be clearly seen developing on the lingual side of the first and second molars after 6 days in culture (Fig. 5 C, E).

To assess whether the supernumerary tooth germs were able to mineralise, the dental field was dissected out from both control and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* embryos at E17.5 and placed into kidney capsules, where they were left to develop for two weeks. To avoid damaging the tooth germs a larger piece of tissue around the molars was dissected which included the neighbouring vestibular lamina. microCT analysis of the kidneys revealed the control tooth germs developed normally, forming a third molar posterior to the second and mineralising appropriately (Fig. 5 F,G). In the mutant samples, multiple tooth germs formed surrounding the main first and second molars on the lingual and labial sides (Fig. 5H,I). The first and second molars themselves had relatively normal development (Figure 5 H, J). Based on the surrounding alveolar bone morphology, with larger and higher located bony lamellae on the labial side, the supernumerary teeth on the labial side (pink) of the first molar were small and conical in shape, while on the lingual side (green) the teeth were larger, more complex with development of cusps (Fig. 5H,J). This may reflect a difference in origin between the labially placed vestibular lamina and the lingually placed RSDL, respectively.

Stimulation of canonical Wnt/β-catenin signalling leads to downregulation of Sox2 in the dental epithelium

In a variety of systems Sox2 has been shown to inhibit Wnt signalling (He et al., 2017; Hashimoto et al., 2012; Mansukhani et al., 2005). Reduction of Wnt signalling has also been shown to lead to upregulation of Sox2 in the lung suggesting the presence of a feedback loop (Ostrin et al., 2018). In order to identify whether altering Wnt signalling has an effect on Sox2 expression in the tooth, we assessed the expression of Sox2 in the tissues where Wnt/β-catenin signalling had been activated. In the control embryos, Sox2 could be seen localised in the nuclei of the RSDL, and dental stalk as expected (Figure 6 A). Immunostaining in the E18.5 *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* embryos however revealed a clear loss of Sox2 in the dental field as well as surrounding oral epithelium (Figure 6 B). This contrasts with a presence of Sox2 positive cells in

other epithelia of the same *Sox2*$^{CreERT2/+;}$*Ctnnb1*$^{lox(ex3)}$ samples, indicating that the effect of Wnt/β-catenin signalling on Sox2 is specific for the dental area (Figure 6C).

To confirm that Wnt/β-catenin signalling has a downregulatory effect on Sox2, we dissected out molar placodes at E14.5 and cultured them in media with either 20uM BIO or DMSO for controls. BIO acts to stimulate the Wnt/β-catenin pathway by inhibiting GSK3β, the kinase which phosphorylates β-catenin to target it for degradation in the destruction complex (MacDonald et al., 2009). After culturing the placodes for two days, there was a marked downregulation of Sox2 compared to the DMSO controls (Figure 6D,E). These data indicated that Wnt/β-catenin signalling negatively regulates *Sox2* expression in the molar.

Removal of the molar leads to revitalisation of the RSDL in culture

The above experiments highlight the potential of the RSDL to form a tooth if given the correct stimulus. This leads to the question of what inhibits Wnt expression in the RSDL in normal development. One possibility is that the first generation tooth inhibits the formation of a successive tooth. For example, in the shrew, where the deciduous teeth are rudimentary, it has been suggested that early activation of the permanent replacement teeth might repress the development of the first teeth (Jarvinen et al., 2008). To test this possibility we dissected slices of the first molar at E16.5, when the RSDL could be clearly observed as a bump on the lingual side of the tooth (Fig. 7 A). The RSDL was then cut from the rest of the tooth and cultured in isolation (Fig. 7 D, E), or the whole tooth slice (molar and RSDL) was cultured intact (Fig. 7 A, B). To allow Wnt signalling to be monitored we used Axin2LacZ reporter mice. In the intact cultures the RDSL was still visible after 4 days of culture as a small protrusion on the side of the first molar (Fig. 7 B, C). Very low levels of canonical Wnt activity were evident in the RDSL as indicated by LacZ staining (blue) after fixation (Fig. 6B). In contrast, the isolated RSDL pieces enlarged and formed cap stage-like tooth germs with upregulated Wnt signalling in the epithelium and mesenchyme (Fig. 7 F,G). Under the confocal microscope a clear cap shape morphology was evident, with LacZ stain in the enamel knot and underlying mesenchyme in 6/10 slices (Fig. 7 H). Using phase contrast, apoptotic bodies were identified in the EK

epithelium, confirming the identity of the structure as a cap stage tooth germ (Fig. 7 I). We repeated the experiment a day later at E17.5. At this stage in isolation a cap-like structure was only observed in 1/10 RSDL pieces, suggesting that the potential of the RSDL to form a tooth has reduced by this time point (data not shown).

Discussion

A long-standing issue in the field of tooth replacement has been what governs whether a tooth is replaced or not. This covers why monophyodonts only have one generation of teeth, but also why most mammals are restricted to two sets. Key to this is the molecular signals that direct whether the dental lamina forms a new tooth or starts to regress. Our results indicate a molecular identity for the parts of the dental lamina that will go on to make a second generation of teeth in diphyodont mammals. In the minipig, we have shown that it is possible to distinguish molecularly between the successional dental lamina and connected interdental lamina, by investigating the expression patterns of Sox2, Sox9 and nuclear β-catenin localisation. The successional lamina, which goes on to form a tooth, does not express Sox2 or Sox9 at its tip, which has high levels of nuclear β-catenin. This restriction of Sox2 from the tip of the odontogenic dental lamina appears conserved across amniotes, the exclusion of Sox2 from this region perhaps being required to maintain Wnt activity.

The rudimentary successional dental lamina (RSDL) in the mouse appears more similar molecularly to the interdental lamina of the minipig, and does not normally have odontogenic potential. The absence of activity canonical Wnt activity in the RSDL is particularly evident. Here we show that activating Wnt signalling in the RSDL in a transgenic mouse leads to proliferation of the RSDL and the formation of a cap stage tooth germ, which can form a mineralised tooth in culture. Similar studies have previously been published, using different modalities of activating the Wnt pathway in oral epithelia. Stabilising β-catenin in a non-inducible K14Cre line (targeting all oral and dental epithelium from

E11.5) led to the formation of multiple epithelial invaginations in both jaws (Järvinen et al., 2006). When transplanted into kidney capsules, these structures formed multiple small teeth, which were able to mineralise. No indication of normal molar development was observed in these mice and this phenotype appears to arise as a consequence of repetitive formation of ectopic tooth germs within the molar placode. In contrast to this, our inducible transgenic mice driven by Sox2Cre produced a subtler phenotype with limited disruption to the first molar, and targeting of the RSDL.

Another study making use of the K14Cre transgenic line with a mosaic pattern of recombination activity showed that the development of supernumeraries is also possible when ablating a restrictive component of the canonical Wnt signalling pathway, adenomatous polyposis coli - APC (Wang et al., 2009). When APC was ablated, β-catenin was able to accumulate in the nucleus and Wnt signalling was therefore constitutively active. This mosaic pattern of Wnt activation led to a phenotype more similar to our mutant embryos, with normally developed main molars, and supernumerary teeth arising from the labial oral epithelium (between the dental and vestibular laminae) as well as from the vestibular lamina itself. Supernumerary teeth can also form around the continuously growing murine incisors throughout adulthood (Wang et al., 2009; Xavier et al., 2015).

Revitalisation of the RSDL appears to involve enhancement of proliferation in the epithelium in this region and was accompanied by condensation of the surrounding mesenchyme and recruitment of blood vessels around the RSDL as a tooth bud started to form. Our *in situ* hybridisation assays showed that the lingual supernumerary tooth germs recapitulate normal gene expression. Tooth germs were also evident in the vestibular lamina. The Sox2+ vestibular lamina develops in intimate connection to the molars (Peterkova et al., 2014) and in a normal situation lacks expression of genes related to tooth development. However like the tooth germs on the lingual side of the tooth, these labial tooth germs formed after activating Wnt signalling were able to produce mineralised teeth. The ability of the vestibular lamina to form teeth may be linked to its

developmental origin, as lineage-tracing experiments have shown that the two laminas (dental and vestibular) form from a common Shh expressing placode (Hovorakova et al., 2016). In patients, odontomas, tooth-like tumours, have been observed to form in the lip furrow region, again suggesting that this tissue has odontogenic potential that can be reawakened in pathological situations (Hovorakova et al., 2016). These results suggest a discreet balance in the Sox2-Wnt/β-catenin network is required for normal development and identity of oral ectodermal organs. Tipping this balance in favour of Wnt/β-catenin can lead to a change in fate of the vestibular lamina to a dental fate.

An interesting observation was the loss of Sox2 protein in the dental field and oral epithelium in the mutant embryos, but not in nasal and soft palate epithelia. This suggests dynamic and tissue-dependent relationships between Sox2 and Wnt signalling. In taste buds and lung Sox2 negatively regulates Wnt expression (Okubo et al., 2006; Hashimoto et al., 2012). In the airway submucosal glands the situation has been shown to be more complex with Sox2 having both inductive and repressive effects on Lef1 depending on the presence of other factors (Xie et al., 2014). In dental tissue Sox2 expression and Wnt activity are complementary, as shown here in the dental lamina, but also in the mouse incisor (Juuri et al., 2012; 2013), suggesting a repressive effect. However, when Sox2 is downregulated in tooth culture using siRNA, Wnt targets are downregulated (Lee et al., 2016), and overexpression of Lef1 can partially rescue the dental defects in conditional Sox2 knockout mice, suggesting that Sox2 can positively regulate Wnt activity (Sun et al., 2016). In addition to the effect of Sox2 on Wnt activity, Wnt activity can impact on Sox2 expression. In the lung reduction of Wnt signalling at E11 led to ectopic Sox2 expression in the epithelium (Ostrin et al., 2018). This effect, however, was highly stage dependent with later loss having no impact on Sox2, even though Sox9 levels were downregulated at both time points. Wnt activity therefore appears to negatively regulate Sox2, but only in some contexts. This agrees with our findings that activating Wnt signalling in culture or in vivo leads to a loss of Sox2 in the dental epithelium, but not other associated epithelia, suggesting a negative feedback loop in action specifically in the dental lamina. This highlights the importance of carefully controlling the

balance between stemness and Wnt-directed differentiation for limited or unlimited tooth formation. It is possible that the Wnt-driven loss of Sox2 in the lamina was enough to stimulate tooth development. This could be investigated further by specifically downregulating Sox2 in the RSDL without altering Wnt signalling.

During mammalian evolution reduction of Wnt signalling at the tip of the successional lamina, may have led to an expansion of Sox2 expression into this region. Presence of Sox2 would have led to further inhibition of Wnt signalling at the tip of the lamina, reinforcing the negative feedback loop and leading to a complete loss of Wnt activity and subsequent loss of odontogenic potential. This would then have been followed by physical loss of the lamina, using a combination of apoptosis and EMT, preventing the possibility of any further tooth replacement (Buchtova et al., 2012).

It has previously been suggested that replacing and successional teeth might inhibit the formation of each other. For example, the successionally developing molars of the mouse inhibit each other, with the second molar forming precociously when isolated from the first molar in culture (Kavanagh et al., 2007). Similarly, the early development of the shrew permanent dentition has been suggested to inhibit the development of the deciduous tooth germs (Jarvinen et al., 2008). Here we show that removal of the first generation tooth in the mouse dramatically frees the RSDL so that it can now form a tooth bud. Given the need for Wnt signalling to initiate tooth development, it is tempting to speculate that a Wnt inhibitor is expressed by the main tooth, inhibiting Wnt activity in the RSDL. Interestingly, the soluble Wnt inhibitors Dickkopf 2 and 3 have been shown to be strongly expressed in the mesenchyme around the developing RSDL at E16 and E18 and may act to inhibit Wnt activity in this region (Fjeld et al., 2005). Restriction of the mouse to one tooth generation could therefore be reliant on the relative positioning and timing of development of the first tooth and the successional lamina.

Although most mammals have two sets of teeth, it may be possible to produce a third set by controlled stimulation of the Wnt pathway. A rudimentary successional lamina from a permanent tooth has been described in both human and bat embryos, suggesting the potential for a third set of teeth (Ooe, 1981;

Popa et al., 2016). Forming a third set, however, would only be feasible, by targeting a small window of time after formation of the replacement dentition, before loss of the dental lamina, and would not be possible later in life.

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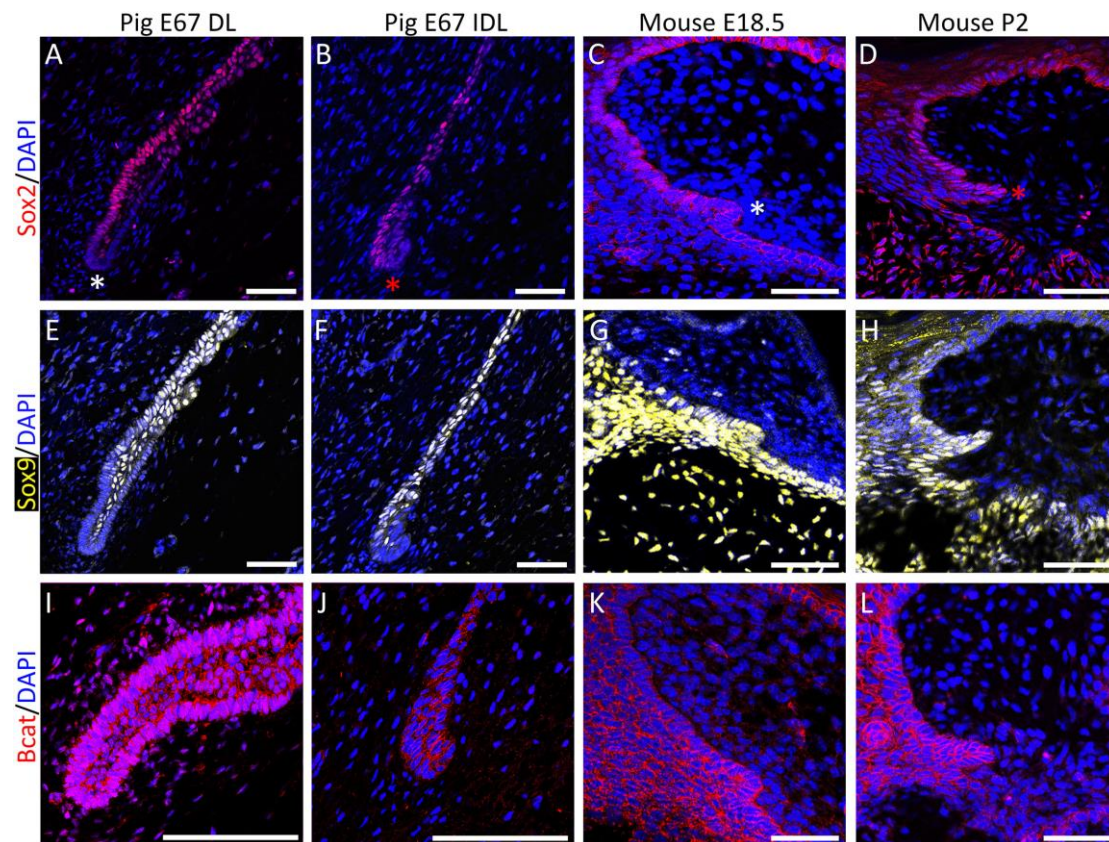
Figures

Figure 1 Molecular similarities and differences between monophyodont RSDL versus diphyodont dental lamina.

Fluorescence immunohistochemistry showing localization of Sox2 (A-D), Sox9 (E-H) and β-catenin (I-L) in the dental (4th premolar) (A,E,I) and interdental (anterior to 4th premolar)(B,F,J) laminae of the miniature pig at 67 days of embryonic development and mouse rudimentary successional lamina at E18.5 (C, G, K) and P2 (D, H, L). β-catenin minipig images are at higher power to highlight the nuclear and cytoplasmic staining. White asterisks: absence of Sox2 in the tip of the miniature pig dental lamina (A) and the mouse RSDL (C). Red asterisks: presence of Sox2 in the tip of the miniature pig interdental lamina (B) and regressing mouse RSDL (D).

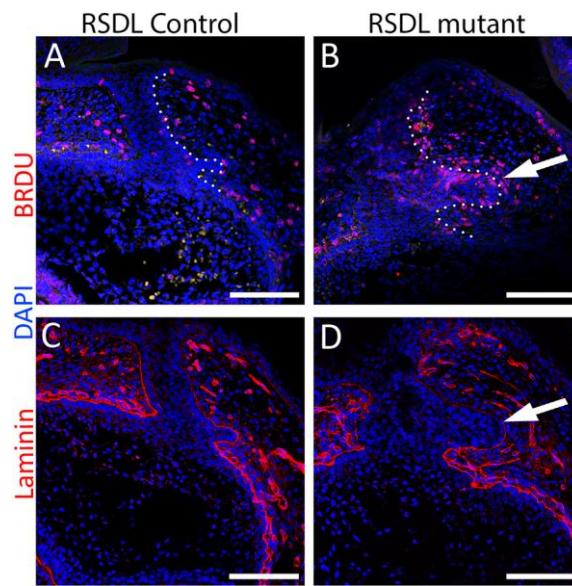DL: dental lamina, IDL: interdental lamina. Scale bars: 50μm.

Figure 2 Localised proliferation and vascularisation associated with the revitalised RSDL

Frontal sections of *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (B,D) and control (*Sox2^CreERT2/-;Ctnnb1^lox(ex3)*) (A,C) embryo RSDLs injected with tamoxifen at E16.0 and collected at E18.5. Fluorescent immunostained for (A,B) BrdU; (C,D) laminin. Proliferation was low in the control RSDL (A) and abundant in the RSDL area in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (white arrow in B). Laminin staining indicates the presence of blood vessels in whorls around the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* RSDL (arrow in D).

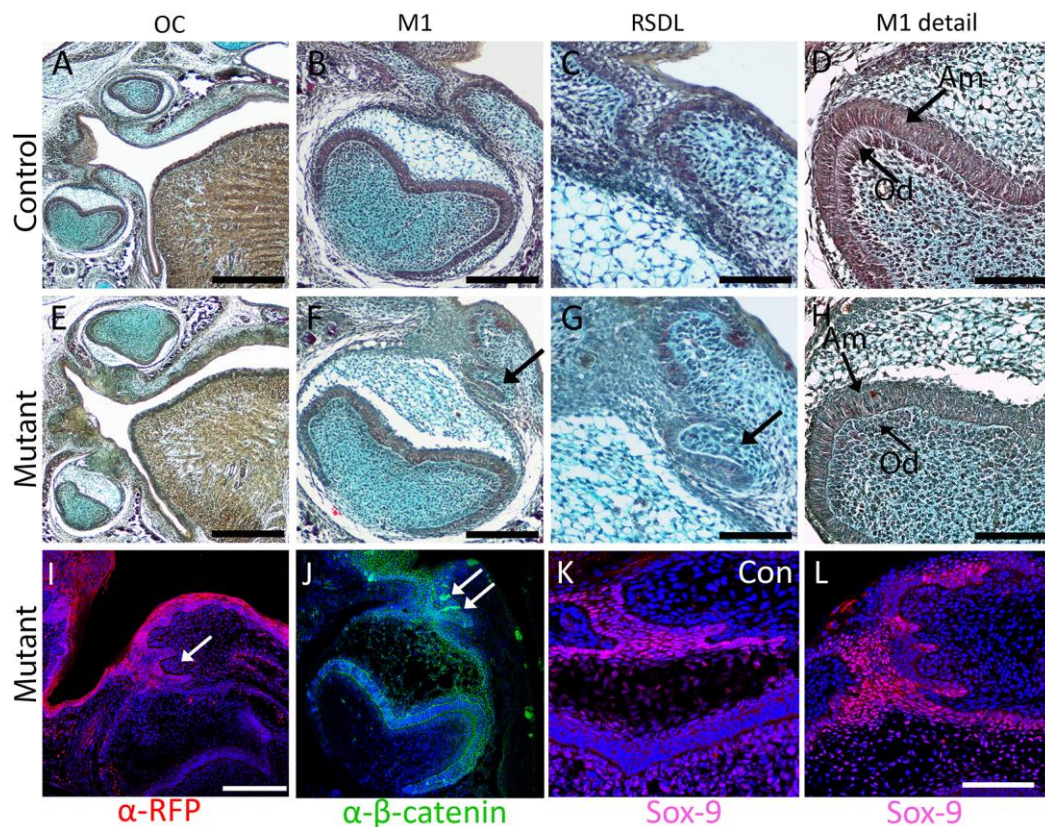White dotted lines delineate dental epithelium; Scale bars 100μm

Figure 3: Stabilising Wnt/ β cat signalling in the Sox2+ cells of the mouse RSDL leads to a novel supernumerary tooth phenotype.

Frontal histological sections stained with a trichrome stain of E18.5 control (A-D) and *Sox2^{CreERT2/+;}Ctnnb1^{lox(ex3)}* (E-H) embryos injected with tamoxifen at E15.5. (A,E) The first molars were largely unaffected, while additional epithelial protrusions were evident in the oral epithelium over the palate. (B,C, F,G) A cap-stage tooth germ formed at the site of the RSDL in the mutant (black arrow). (D,H) In the *Sox2^{CreERT2/+;}Ctnnb1^{lox(ex3)}* samples, the odontoblasts appeared less organised without the normal columnar appearance (arrows in D, H). (I) Lineage tracing of cells in which Wnt signalling was stabilised. Arrow points to the supernumerary tooth germ with is positive for Red Fluorescent protein. (J) Nuclear β-catenin is evident in clusters of cells within the supernumerary tooth

germ. (K,L) Sox9 immuno in control (K) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* embryos at E18.5.

OC: oral cavity; M1: first molar; M2: second molar, Am: ameloblasts, Od: odontoblasts. Scale bars A, E 500 µm; B, F, I, J 200 µm; C, G, D, H, K, L 100µm.
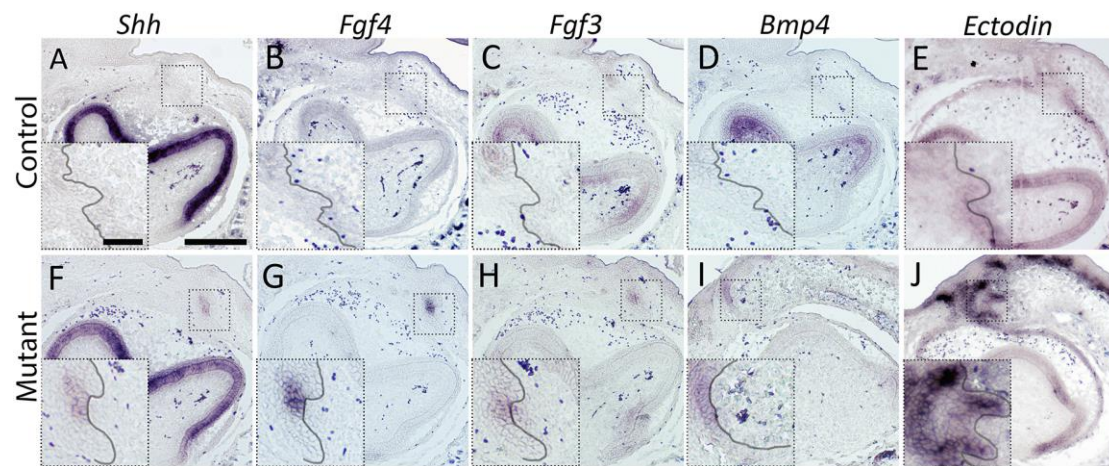
Figure 4 Supernumerary tooth germs express odontogenic markers

In situ hybridisation for genes of interest in E18.5 control (A-E) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (F-J) embryo first molars, injected at E15.5. (A,F) *Shh* was expressed in the inner enamel epithelium of both control and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* first molars but only present in the mutant supernumerary tooth germ. (B-D, G-I) *Fgf4*, *Fgf3* and *Bmp4* are absent in the control RSDL but mark the enamel knot area of the supernumerary tooth germ. Expression of *Fgf3* and *Bmp4* in the odontoblast layer in the mutant M1 are reduced. (E, J) *Ectodin* is expressed strongly in the supernumerary tooth germ, but excluded from the enamel knot, as in WT tooth development. Dotted square in each image shown as an inset bottom left hand side.

Scale bars in (A) 25µm (inset) and 100µm. Same scale in all other images.

Control Mutant

A

Ab
La
A ← → P
Li
O

Ab
Li
A ← → P
La
O

La
O
A ← → P
Ab
Li

Control Mutant

B   C

E17.5

D   E

E17.5+6

F   G

Control

H

I   J

Mutant

Development • Accepted manuscript

Figure 5 Supernumerary tooth germs mineralise and form roots

(A) 3D reconstructions show the phenotype is restricted on the lingual side at E18.5. Three-dimensional reconstructions of serial histological sections showing the control and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* tooth germs from lingual, labial and oral perspectives. The area highlighted in grey shows the supernumerary tooth germs that formed on the lingual side of the dental lamina of the first molar. A: anterior, P: posterior; O: oral, Ab: aboral, La: labial, Li: lingual.

(B-E) Development of supernumerary teeth in explant culture. (B,D) Control and (C,E) *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* tooth germs from embryos injected at E15.5 and collected at E17.5, then cultured for six days in an explant culture system (Control N=3, *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* N=5). Red fluorescence: tomato protein. Scale bar in B 500μm (same scale in C-E).

(F, I) Paraffin sections of control (F) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (I) teeth cultured under a kidney capsules and stained with trichrome. (Control N=2, *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* N=3) (G, H,J) 3D reconstructions from microCT scans showing hard tissues of control (G) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* teeth (H,I). (G) Control culture show three molars (M1 far right). (H) *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* reconstruction with the main molars (yellow) surrounded on the lingual side (green) and labial side (pink) with mineralised teeth. (J) Segmented view of *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* molars and lingual teeth, shown from a root and crown view, highlighting multiple cusps of lingual teeth next to the first molar.

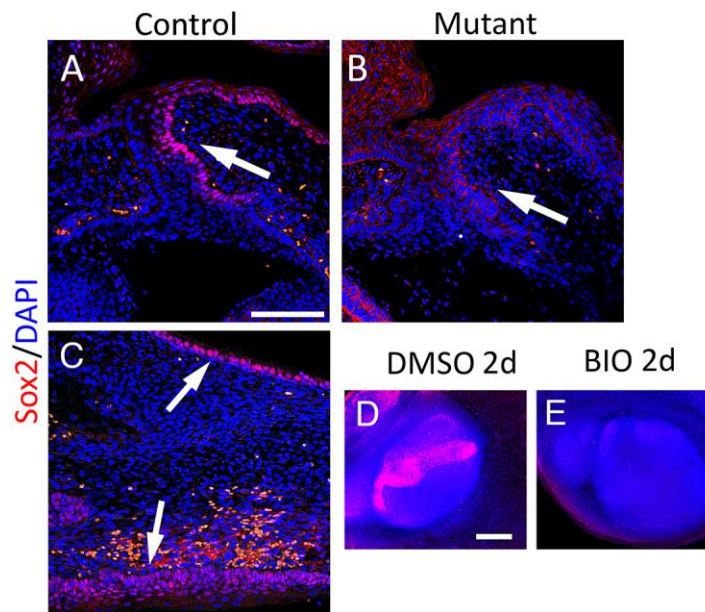Scale bars: F = 200 μm, I = 500 μm, G = 0.5mm (same scale in J)

Figure 6 Wnt/β-catenin activation leads to Sox2 mRNA and protein downregulation

(A, B, C) Fluorescence immunohistochemistry for Sox2 in wax sections of E18.5 embryos. (A) Control RSDL. Arrow in A points to nuclear localisation of Sox2 in the RSDL and lingual stalk. (B,C) *Sox2$^{CreERT2/+;}$Ctnnb1$^{lox(ex3)}$* epithelial tissues. (B) *Sox2$^{CreERT2/+;}$Ctnnb1$^{lox(ex3)}$* RSDL. Arrow in B points to lack of nuclear Sox2 in the *Sox2$^{CreERT2/+;}$Ctnnb1$^{lox(ex3)}$* RSDL area (C) *Sox2$^{CreERT2/+;}$Ctnnb1$^{lox(ex3)}$* nasal and palate epithelium. Arrows in C point to nuclear localisation of Sox2 in these epithelia. (D, E) Fluorescence immunohistochemistry for Sox2 in whole mount explants of E14.5 wild type embryo dental tissues, cultured in either DMSO or BIO for two days. (Control N=3, BIO N=4). Scale bars in A and D 100μm, same scale in B, C, E.
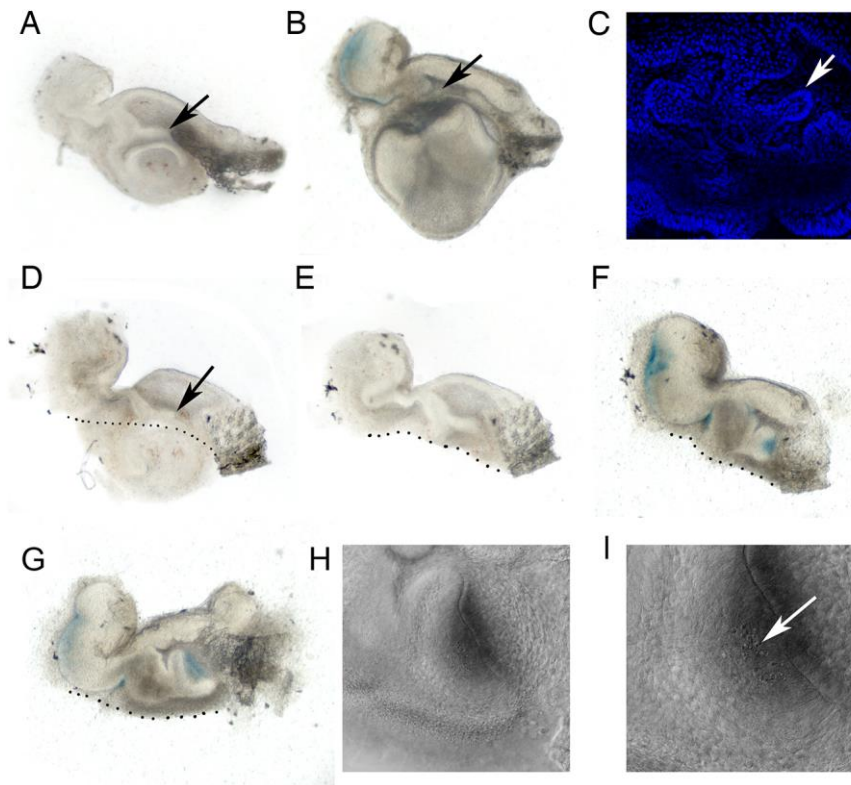
Figure 7

Repression of the RSDL by the first tooth generation

(A) E16.5 first molar tooth germ slice. Lingual to the right, buccal to the left in all images. Arrow point to rudimentary successional dental lamina (RSDL). (B) Same slice as in (A) after 4 days in culture, fixed and stained for Axin2 expression. The arrow points to the RSDL, which have very low levels of stain (blue). The buccal epithelium of the cheek in contrast shows high levels of Axin2 expression. (C) Same slice viewed under confocal, nuclei stained with DAPI. (D) E16.5 first molar tooth germ slice. Arrow point to rudimentary successional dental lamina (RSDL), dotted line indicates level of cut. (E) Same slice as in (D) after cut (dotted line) and cultured for 1 day. (F) Same slice after 4 days in culture, fixed and stained for Axin2 expression (blue). As before the buccal cheek epithelium is positive but so is the forming cap stage tooth germ on the lingual side. (G-I) Another example of an isolated RSDL after 4 days in culture. (G) Dotted line show cut. A clear cap stage tooth germ has developed. (H) High power of the tooth germ highlighting Axin2LacZ stain in the forming enamel knot and underlying mesenchyme. (I) Enamel knot area under phase contrast to highlight the apoptotic bodies (arrow) in the epithelium. Cultures N = 10 intact and N = 10 cut.
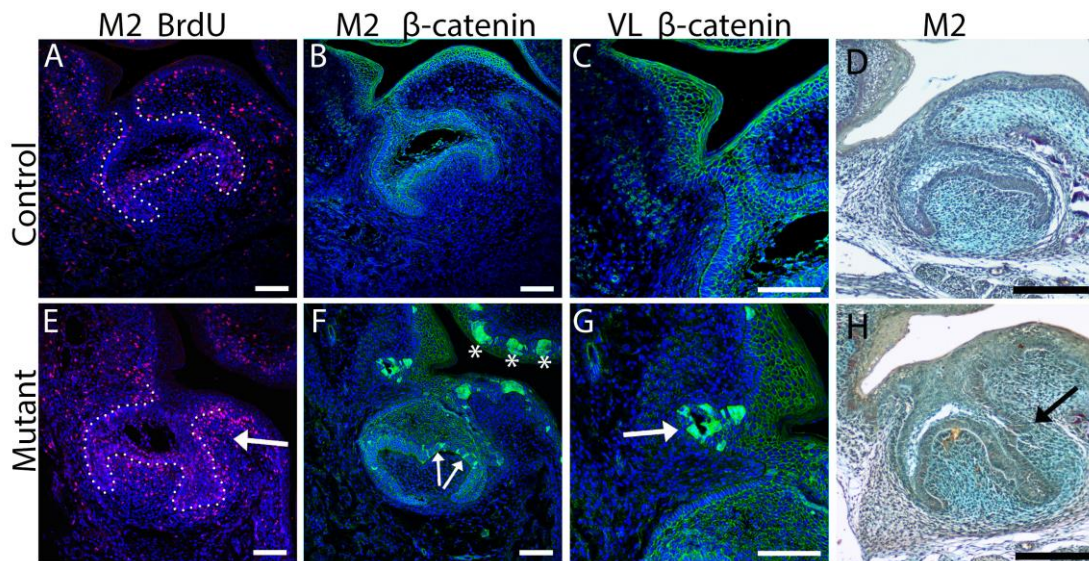
Figure S1.

The lingual side of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* **second molar is proliferative and forms protrusions**

Frontal sections of control (*Sox2^CreERT/--;Ctnnb1^lox(ex3)*)(A-D) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (E-H) embryo second molar injected with tamoxifen at E15.5 and collected at E18.5.

(A, E) fluorescence immunostaining for BrdU; proliferation was low in the control and abundant in the lingual area of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (white arrow in E); white dotted lines delineate dental epithelium. (B, C, F, G) fluorescence immunostaining for β-catenin; nuclear staining was absent in control, but present in distinct patches in the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* oral epithelium (asterisks in F), lingual side and inner enamel epithelium of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* second molar (arrows in F) and vestibular lamina (arrow in G). (D,H) Histology of M2, arrow in H showing protrusion on lingual side.

M2: second molar; VL: vestibular lamina. Scale bars A, B, C, E, F, G 100 μm; D, H: 200 μm.
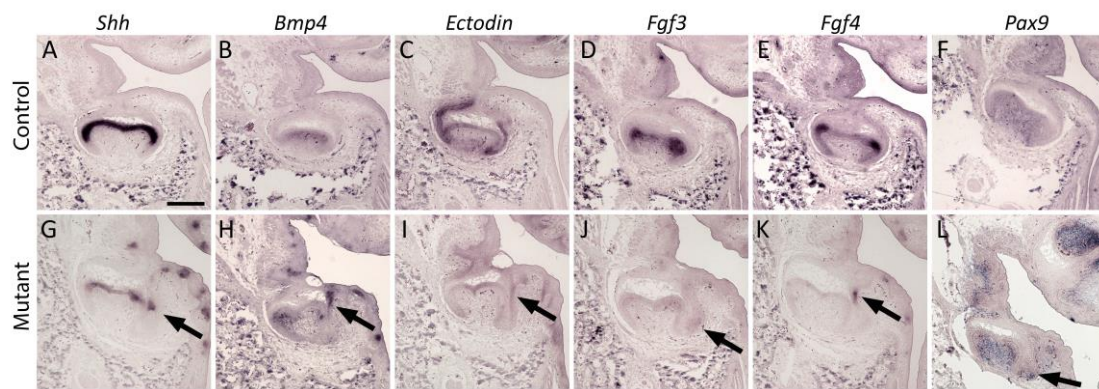
Figure S2.

The lingual side of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* **second molar expresses odontogenic markers**

In situ hybridisation for *Shh* (A, G), *Bmp4* (B, H), *Ectodin* (C, I), *Fgf3* (D, J), *Fgf4* (E, K), *Pax9* (F, L) in E18.5 control (A-F) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (G-L) embryonic second molars injected at E15.5. All genes were expressed as expected in the control molars as well as the main body of the mutant second molars: *Shh* marked the inner enamel epithelium, *Bmp4* was expressed in the papilla mesenchyme, *Ectodin* in the inner and outer enamel epithelia, *Fgf3* in the secondary enamel knots and underlying mesenchyme, *Fgf4* in the secondary enamel knots and *Pax9* in the papilla mesenchyme; arrows indicate ectopic areas of gene expression in the lingual protrusions of the *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* molars. Scale bar 200μm.

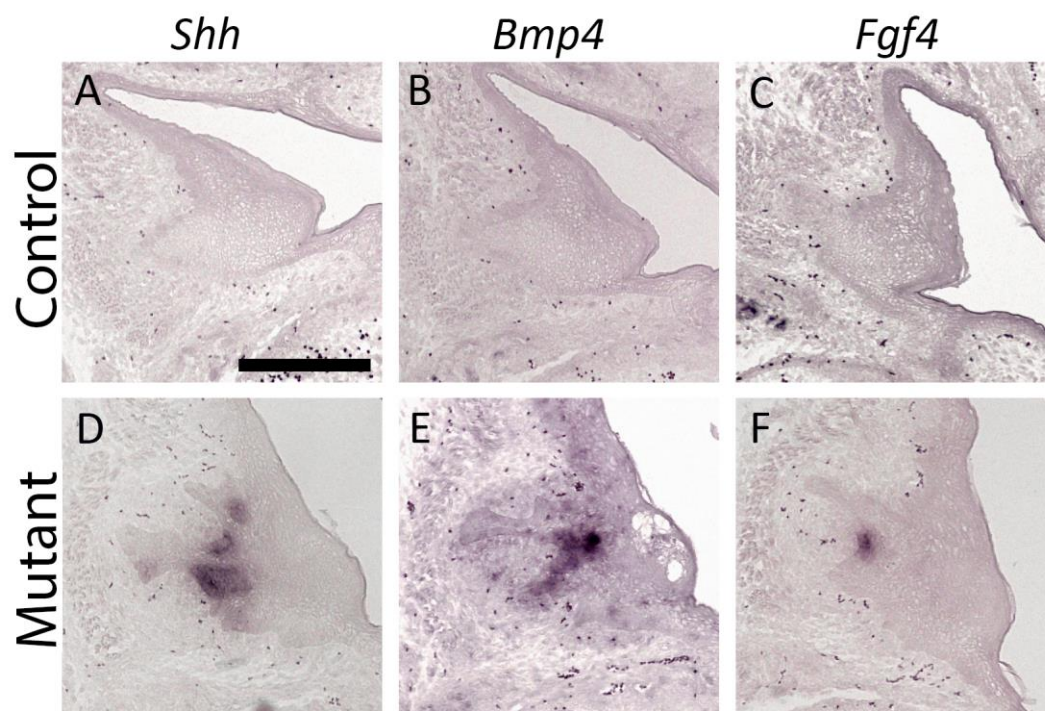**Figure S3. The vestibular lamina retains odontogenic capacity**

In situ hybridisation in E18.5 control (A-C) and *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* (D-F) vestibular lamina of embryos injected at E15.5. None of the genes are expressed in the control vestibular lamina (A-C). Arrows point to patches of expression of *Shh* (D), *Bmp4* (E) and *Fgf4* (F) in the vestibular laminae of *Sox2^CreERT2/+;Ctnnb1^lox(ex3)* embryos.

Scale bar in A 200μm, same scale in other images.