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Signaling by FGFR2b controls the regenerative capacity of adult mouse incisors

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SUMMARY

Rodent incisors regenerate throughout the lifetime of the animal owing to the presence of epithelial and mesenchymal stem cells in the proximal region of the tooth. Enamel, the hardest component of the tooth, is continuously deposited by stem cell-derived ameloblasts exclusively on the labial, or outer, surface of the tooth. The epithelial stem cells that are the ameloblast progenitors reside in structures called cervical loops at the base of the incisors. Previous studies have suggested that FGF10, acting mainly through fibroblast growth factor receptor 2b (FGFR2b), is crucial for development of the epithelial stem cell population in mouse incisors. To explore the role of FGFR2b signaling during development and adult life, we used an rtTA transactivator/tetracycline promoter approach that allows inducible and reversible attenuation of FGFR2b signaling. Downregulation of FGFR2b signaling during embryonic stages led to abnormal development of the labial cervical loop and of the inner enamel epithelial layer. In addition, postnatal attenuation of signaling resulted in impaired incisor growth, characterized by failure of enamel formation and degradation of the incisors. At a cellular level, these changes were accompanied by decreased proliferation of the transit-amplifying cells that are progenitors of the ameloblasts. Upon release of the signaling blockade, the incisors resumed growth and reformed an enamel layer, demonstrating that survival of the stem cells was not compromised by transient postnatal attenuation of FGFR2b signaling. Taken together, our results demonstrate that FGFR2b signaling regulates both the establishment of the incisor stem cell niches in the embryo and the regenerative capacity of incisors in the adult.

KEY WORDS: Fibroblast growth factor receptor 2b, FGF10, Enamel, Ameloblasts, Stem cells, Incisor regeneration, Mouse

INTRODUCTION

The continuous growth of rodent incisors throughout life results from the activity of stem cells located at their proximal end. Incisor growth is counteracted by abrasion during feeding leading to a fixed length of the tooth. Enamel, the hardest substance in the tooth, is present on the labial (lip), but not lingual (tongue), side of the tooth because the enamel-producing ameloblasts are only produced on the labial surface. This asymmetric enamel deposition leads to preferential abrasion on the lingual surface.

The incisor epithelial stem cells reside in distinct anatomical structures called cervical loops (CLs). Each incisor has two CLs, one located on the labial and lingual aspect, respectively. The CLs are epithelial structures consisting of a central core of stellate reticulum (SR) cells surrounded by the columnar epithelial cells of the inner and outer enamel epithelia (IEE and OEE, respectively; Fig. 1A). It is thought that epithelial stem cells are located in either the OEE or the stellate reticulum at the apex of the CLs (Harada et al., 1999). Stem cells in the CLs self-renew and give rise to proliferating progenitors known as transit-amplifying (T-A) cells (Fig. 1A). On the labial side of the incisor, the T-A cells give rise to pre-ameloblasts that proliferate and differentiate into ameloblasts as they migrate distally (Harada et al., 1999; Wang et al., 2007). By contrast, the progeny of the stem cells on the lingual side have not yet been described, nor have the mesenchymal stem cells been identified.

Tooth development provides an excellent model for studying interactions of epithelium and mesenchyme during organogenesis. During embryogenesis, incisor development starts with the formation of a placode from the oral ectoderm at embryonic day (E) 12. Over the next 2 days, the placode expands to form a bud that invades the underlying mesenchyme. By E15, the developing incisor has formed CLs on both the labial and lingual sides. During the next 3 days, the incisor continues to expand and will form mature ameloblasts, enamel, dentin and pre-dentin on the labial side (Kerley, 1975). Factors such as notch, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) play important roles during tooth development (Wang et al., 2007). In particular, FGFs are crucial growth factors for both incisor and molar development. In molars, mesenchymal FGF3 and FGF10 signal through epithelial fibroblast growth factor receptor 2b (FGFR2b). FGF10 also maintains sonic hedgehog (*Shh*) expression, which marks cells that are differentiating along the ameloblast lineage (Bitgood and McMahon, 1995; Klein et al., 2008).

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In the incisors, *Fgf3* and *Fgf10* are expressed in the mesenchymal cells adjacent to the IEE, and *Fgf10* is also expressed in the mesenchyme surrounding the CL. FGF10 binds to FGFR2b, which is expressed in the epithelium of the CL (Harada et al., 1999). Owing to the perinatal lethality of *Fgfr2b*-null embryos, it has not been possible to study the role of FGFR2b ablation in postnatal incisors (De Moerlooze et al., 2000). *Fgf10*-null embryos have hypoplastic incisors, and in vitro culture of developing incisors in the presence of FGF10-blocking antibodies causes regression of the labial CL, suggesting that FGF10 acts as a survival factor for stem cells in the CL (Harada et al., 2002). It has been reported that in the incisors of $Fgf10^{-/-}$ mice, a root analog forms on the labial side as a result of cessation of proliferation in the IEE and increased proliferation in the OEE (Yokohama-Tamaki et al., 2006). Thus, cessation of *Fgf10* signaling might trigger the transition from crown to root.

More recently, epithelial-specific deletion of *Fgfr2* in the CL using the $Nkx3.1^{Cre}$ driver line suggested that FGFR2 signaling is required for the development and maintenance of the maxillary CL (Lin et al., 2009). However, this experiment did not address the role of FGFR2b during later stages of embryonic development and adult homeostasis of the incisor, as $Nkx3.1^{Cre}$ is already active at E11.5 in the epithelium of the developing incisor. To circumvent the perinatal lethality of homozygous *Fgfr2b* mutants, we previously developed a mouse model allowing inducible and reversible attenuation of FGFR2b signaling using the rtTA transactivator/tetracycline promoter system (Parsa et al., 2008). Mice expressing rtTA under control of the ubiquitous *Rosa26* promoter were crossed with *tet(O)sFgfr2b* mice, which carry a transgene encoding a dominant-negative soluble FGFR2b. Administration of doxycycline to the double-transgenic (DTG) heterozygous offspring of this cross leads to ubiquitous expression of the dominant-negative receptor. Using this model, we have analyzed the role of FGFR2b during different stages of embryonic development and homeostasis of the incisors. Attenuation of FGFR2b signaling from early stages of embryonic incisor development led to the formation of a rudimentary CL with a reduced pool of ameloblast progenitors. Blockade of FGFR2b signaling from postnatal day (P) 14 onwards caused an almost complete loss of the maxillary incisor and deficient enamel deposition in the mandibular incisor. However, release of inhibition of FGFR2b signaling allowed incisor growth to resume normally, indicating that under our experimental conditions, FGFR2b signaling is not essential for the maintenance of the stem cells in adult mice. This model allows us to determine the cellular mechanisms controlled by FGFR2b signaling both during development in the embryo and during homeostasis of the incisors in adult mice.

MATERIALS AND METHODS

Generation of *rtTA; tet(O)sFgfr2b* animals

CMV-Cre mice (Schwenk et al., 1995) were crossed with $rtTA^{flox}$ mice (Belteki et al., 2005) to generate mice expressing rtTA under the ubiquitous *Rosa26* promoter. This constitutive rtTA mouse line was then crossed with the *tet(O)sFgfr2b* responder line (Hokuto et al., 2003) to generate DTG heterozygous animals, allowing ubiquitous expression of dominant-negative soluble FGFR2b (Gossen and Bujard, 1992). All mice were generated on a CD1 mixed background. Inducible and reversible attenuation of the FGFR2b pathway was achieved by administration of doxycycline-containing food [normal rodent diet with 0.0625% doxycycline (Harlan Teklad)]. Mice were genotyped as described previously (Schwenk et al., 1995; Hokuto et al., 2003; Belteki et al., 2005). Five animals were used for each control and experimental group. Adult DTG animals not exposed to doxycycline were phenotypically undistinguishable from control mice (Parsa et al., 2008). The control group consisted of wild-type or single-transgenic animals from the same litter. Animal experiments were performed under the research protocol approved by the Animal Research Committee at Children's Hospital Los Angeles and at Osaka Dental University.

$Fgf10^{lacZ}$ animals

This reporter line has been described previously (Mailleux et al., 2005). In this line, an *nlacZ* transgene is inserted upstream of the *Fgf10* gene.

Tissue preparation and histology

Embryonic heads were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Jaws of control and DTG animals were collected and fixed in 4% PFA and 0.2% picric acid in PBS overnight at 4°C. Jaws of postnatal animals were decalcified in 5% EDTA containing 4% sucrose in PBS for 2 weeks at 4°C. Samples were dehydrated in a graded ethanol series, paraffin-embedded and 5 μm sections were prepared. Sections were deparaffinized and either stained with Hematoxylin and Eosin or used for immunohistochemistry or in situ hybridization.

Immunohistochemistry

Deparaffinized sections were washed in 3% H_2O_2 in methanol for 10 minutes at room temperature. Antigen retrieval was performed in citrate buffer (pH 6) at 95°C for 15 minutes. Sections were incubated with primary antibodies at 4°C overnight. The primary antibodies used were: anti-amelogenin (1:5000; Santa Cruz), anti-FGFR1 (1:1000; Santa Cruz), anti-FGFR2 (1:500; Santa Cruz), anti-keratin 14 (1:500; Thermo Scientific), anti-Ki67 (1:100; Thermo Scientific) and anti-E-cadherin (1:1000; BD Biosciences). Immunohistochemistry was performed with SuperPicture (Invitrogen) or Dako EnVision (Dako) kits followed by counterstaining with Hematoxylin. Images were taken using a Leica color camera attached to a Leica DM4000B microscope.

Cell proliferation assays

A commercially prepared pre-diluted solution of 5-bromo-2-deoxyuridine (BrdU; catalog number 00-0103, Invitrogen) was injected intraperitoneally (1 ml per 100 g body weight) 2 hours before sacrifice. Incorporated BrdU was detected using the Zymed BrdU Staining Kit (Invitrogen). Sections from maxillary and mandibular incisors were analyzed from three independent control and three DTG animals. First, the area of the CL was quantified for each section, followed by quantification of the number of proliferating cells within the CL area. For each section, the results were presented as a ratio of the number of proliferating cells to the area of the CL. Statistical significance of the difference between control and DTG animals was evaluated by a one-tailed paired *t*-test. $P<0.05$ was considered statistically significant.

In situ hybridization

In situ hybridization was carried out as described previously (de Maximy et al., 1999).

Quantitative RT-PCR

RNA was extracted from microdissected tissue containing the cervical loop region of mandibular incisors from doxycycline-treated DTG mice or untreated age-matched controls. Random hexamers (Roche) and M-MLV reverse transcriptase (Promega) were used for reverse transcription. All quantitative PCR (qPCR) reactions were performed using the GoTaq qPCR Master Mix (Promega) in a Mastercycler Realplex (Eppendorf); each reaction was run in triplicate. Primers were designed using the Primer Blast online tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences were (FW, forward; RW, reverse; 5′-3′): Etv4-FW, AAGGCGGATACTTGGACCAG; Etv4-RW, GCCACGTCTCTTGGAAGTGA; Etv5-FW, TAACGACTGGTCACCTGCTGGGGA; Etv5-RW, AACGGCTGTGCAGGGTCCAAA; Gapdh-FW, AGGTCGGTGTGAACGGATTTG; Gapdh-RW, TGTAGACCATGTAGTTGAGGTCA; Notch1-FW, CAGGCTCCAGTGTTCTGTAG; Notch1-RW, CAGTCCGTCTGTCCTCAGTT; Notch2-FW, GTGGACGTGCTGGACGTGAA; Notch2-RW, ATCTCGCCAGTGCGGTCTGT; Shh-FW, CTGCCATCGCAG-

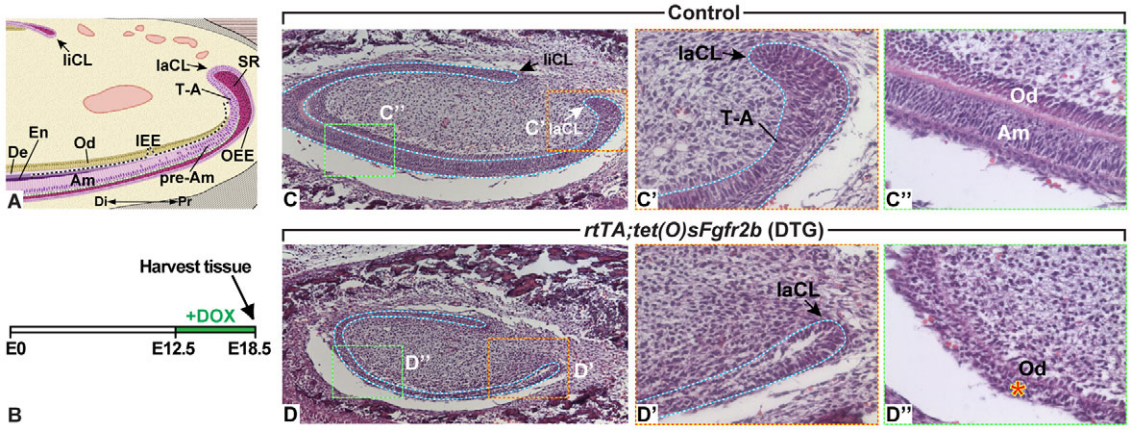

Fig. 1. Attenuation of FGFR2b signaling during mid- and late embryonic development impairs ameloblast formation and cervical loop morphology. (**A**) Schematic of a sagittal section through the proximal region of the mouse incisor. The lingual and labial cervical loops (liCL and laCL, respectively) are believed to contain epithelial stem cell niches. Only stem cells in the laCL give rise to enamel (En)-producing ameloblasts (Am), which abut the mesenchymally derived dentin (De)-secreting odontoblasts (Od). Stem cells in the laCL, which are thought to reside in the stellate reticulum (SR) core or the outer enamel epithelium (OEE), produce transit-amplifying (T-A) cells in the inner enamel epithelium (IEE), which, in turn, give rise to pre-ameloblasts (pre-Am) that develop into mature ameloblasts. The IEE (black and white dotted bracket) includes the T-A cells, the pre-ameloblasts and the ameloblasts. (**B**) Timecourse for doxycycline (DOX) treatment of females carrying *rtTA;tet(O)sFgfr2b* (DTG) and single-transgenic control embryos. (**C-D″**) Hematoxylin and Eosin staining of sagittal sections through the proximal incisor of control (C-C″) and DTG embryos (D-D″) at E18.5. C′, C″, D′ and D″ are magnifications of the areas indicated in C and D. Asterisk in D″ represents missing columnar cells in DTG embryos. Blue dotted lines outline the epithelium. Di, distal; Pr, proximal.

CCCCAGTC; Shh-RW, TGCGTGTGCGCTCCTCCTTG; Spry2-FW, CCTCTGTCCAGGTCCATCAGCACTGTCAGC; Spry2-RW, GCAGC-AGCAGGCCCGTGGGAGAAG. Expression levels of the genes of interest were normalized to levels of *Gapdh* and are presented as levels relative to untreated controls.

MicroCT analysis

Images of microCT analysis of 6-week-old control and DTG mice were obtained using a microfocus X-ray CT system (SMX-130CTSV3, Shimadzu). Scans were performed at 95 kV, with an electric current of 4 msA, with a brass filter, in 27.5 μm-thick layers and a field of view (*xy*) of 14.091 mm. The resolution of computed tomography tomogram slices was 512×512 pixels. Three-dimensional images were constructed with three-dimensional image visualization software (VG Studio MAX 1.2, Nihon Visual Science) for evaluating the differences in enamel formation in each sample.

X-Gal staining

For X-Gal staining, jaws were dissected from freshly euthanized animals and the bone covering the proximal incisor was removed. Following fixation at 4°C in 100 mM phosphate buffer containing 2% PFA, 5 mM EGTA, 0.2% glutaraldehyde and 2 mM $MgCl_2$, the tissue was washed in 100 mM phosphate buffer with 0.01% sodium deoxycholate and 0.02% Nonidet P40. Staining was performed at 37°C overnight using the washing solution described above with 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide and 1 mg/ml X-Gal added. The specimens were washed, fixed in 4% PFA overnight, decalcified and further processed for paraffin sectioning. Sections were counterstained with Hematoxylin and mounted using Permount (Fisher Scientific).

RESULTS

Ubiquitous inducible expression of soluble FGFR2b phenocopies the inactivation of *Fgfr2b* expression

We tested first whether our DTG system leads to inhibition of FGFR2b by inducing the expression of soluble FGFR2b from E9.5-18.5 (see Fig. S1 in the supplementary material). We found that DTG embryos exposed to doxycycline during this time phenocopy $Fgfr2b^{-/-}$ embryos (De Moerlooze et al., 2000). Additionally, DTG embryos not exposed to doxycycline (see Fig. S1A,B in the supplementary material), as well as single-transgenic embryos exposed to doxycycline (data not shown), were identical to wild-type embryos. This result demonstrates that there is no leakiness in the expression of soluble FGFR2b in our DTG embryos in the absence of doxycycline, and that doxycycline does not adversely affect embryonic development. DTG embryos exposed to doxycycline from E9.5-18.5 exhibited limb agenesis (see Fig. S1C in the supplementary material), absence of eyelid closure (see Fig. S1C in the supplementary material), and cleft palate (see Fig. S1D in the supplementary material). In addition, we observed a curly tail in DTG embryos (see Fig. S1C in the supplementary material), which is a phenotype observed in $Fgfr2b^{-/-}$ (De Moerlooze et al., 2000) but not in $Fgf10^{-/-}$ (Harada et al., 2002) embryos. Together, these results indicate that our mouse model allows specific and robust inhibition of FGFR2b signaling in an inducible and non-leaky fashion.

Attenuation of FGFR2b signaling during development leads to the formation of a rudimentary cervical loop

In order to gain insight into the role of FGFR2b signaling in regulation of the development of mandibular and maxillary incisors, pregnant females carrying DTG and control embryos were exposed to doxycycline from E12.5-18.5 (Fig. 1B) and incisors of E18 embryos were analyzed. The control embryos exhibited well-formed mandibular and maxillary incisors with clearly distinguishable CLs on the labial and lingual aspects (Fig. 1C,C′; data not shown). Higher magnification images showed the presence of well-organized ameloblasts and odontoblasts (Fig. 1C″). By contrast, in the DTG embryos, the CLs and differentiated cell types in both mandibular and maxillary incisors were abnormal (Fig. 1D-D″; data not shown). DTG mandibular incisors exhibited a rudimentary labial CL similar in shape to the lingual CL observed

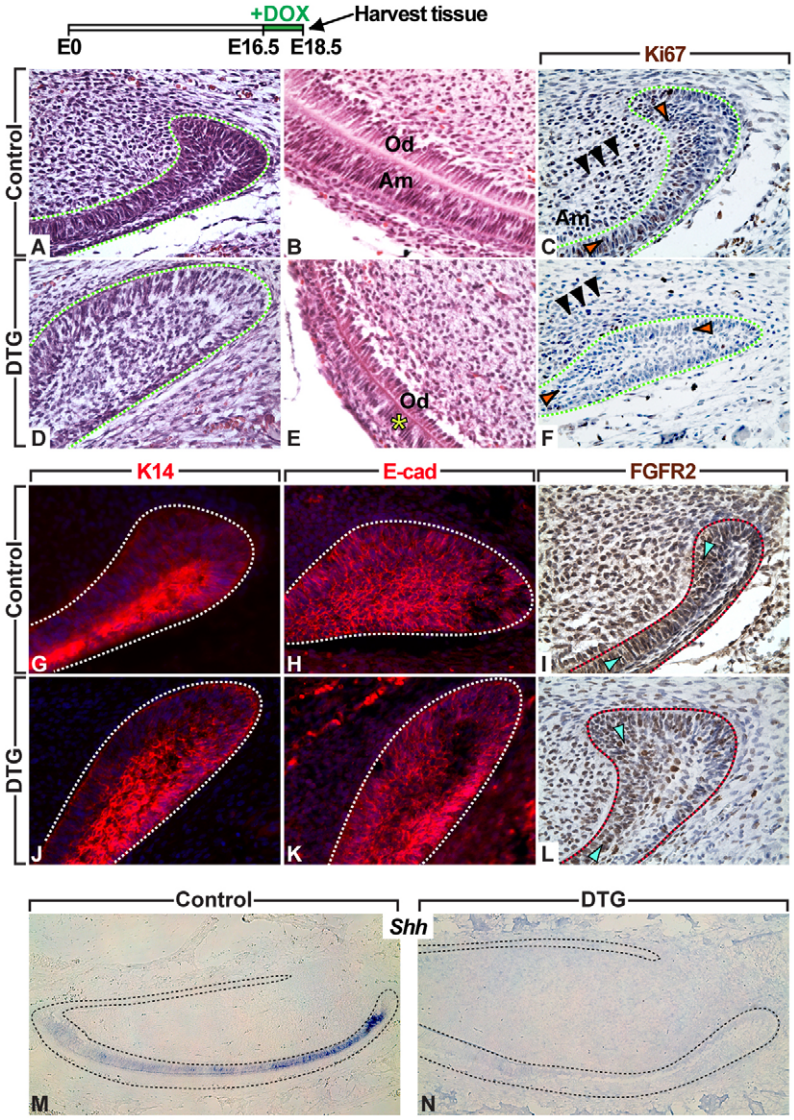

Fig. 2. Decreased FGFR2b signaling during late incisor development leads to reduction in T-A cells, impaired amelogenesis and cervical loop expansion. (**A-N**) Hematoxylin and Eosin staining (A,B,D,E), analysis of cell proliferation (C,F), immunohistochemistry (G-L) and *Shh* in situ hybridization (M,N) of E18.5 control (A-C,G-I,M) and DTG embryos (D-F,J-L,N) after 48 hours exposure to doxycyline (treatment timecourse shown at the top). Red arrowheads in C and F delimit the approximate T-A cell regions in control and DTG embryos, respectively, and black arrowheads indicate mesenchymal proliferation. Asterisk in E represents abnormally short, less organized ameloblasts. Note that FGFR2 is most highly expressed in the T-A cells (region between blue arrowheads in I and L). Dotted lines outline the epithelium. Am, ameloblasts; Od, odontoblasts.

in controls (compare Fig. 1D′ with 1C). In DTG incisors, the layer normally comprising tall, columnar ameloblasts was markedly abnormal, consisting of short, cuboidal cells (Fig. 1D″). This phenotype is very similar to that previously reported for homozygous *Fgf10* mutants (Yokohama-Tamaki et al., 2006).

FGFR2b signaling plays a crucial role in development of epithelial cells in the cervical loop and inner enamel epithelium of mandibular incisors

In order to investigate the role of FGFR2b signaling in the maintenance of epithelial cells during late incisor development, pregnant females carrying DTG and control embryos were treated with doxycycline from E16.5-18.5, which is after morphogenesis of the incisors is largely complete. Histological analysis of sagittal sections of the mandibular incisors demonstrated that, compared with control littermates (Fig. 2A), the labial CL of the DTG embryos was enlarged and was missing the typical T-A region (Fig. 2D). In more mature regions, the ameloblasts were disorganized and appeared less columnar in DTG incisors (Fig. 2E) compared with the control (Fig. 2B). In addition, cell proliferation was markedly decreased in the epithelium of both mandibular (Fig. 2F) and maxillary (data not shown) incisors of the DTG embryos compared with control incisors (Fig. 2C). Interestingly, proliferation was still detected in the mesenchymal cells of the DTG incisors (Fig. 2F), indicating a specific role for FGFR2b signaling in the regulation of cell proliferation in the epithelial cells.

To characterize further the status of the cells in the labial CL of DTG embryos, immunofluorescence for keratin 14 (K14) and E-cadherin (cadherin 1 – Mouse Genome Informatics), two markers of epithelial cells, was performed. K14 was highly expressed in the SR-containing core region of the labial CL in controls (Fig. 2G) and this area of high K14 expression was expanded in the labial CL of the DTG embryos (Fig. 2J), reflecting enlargement of the labial CL. By contrast, E-cadherin expression appeared to be unchanged (Fig. 2H,K). Finally, antibodies against the tyrosine kinase domain of FGFR2 were used to detect the expression of FGFR2b, the major receptor for FGF10. These antibodies detect both the FGFR2c isoform, which is expressed in the mesenchyme, and the FGFR2b isoform, which is expressed in the epithelium. In the control incisor, the T-A domain of the labial CL was strongly positive for FGFR2b, whereas expression in the epithelium at the apex of the CL appeared to be more heterogeneous. Similar expression was observed in the DTG incisor and, in addition, there

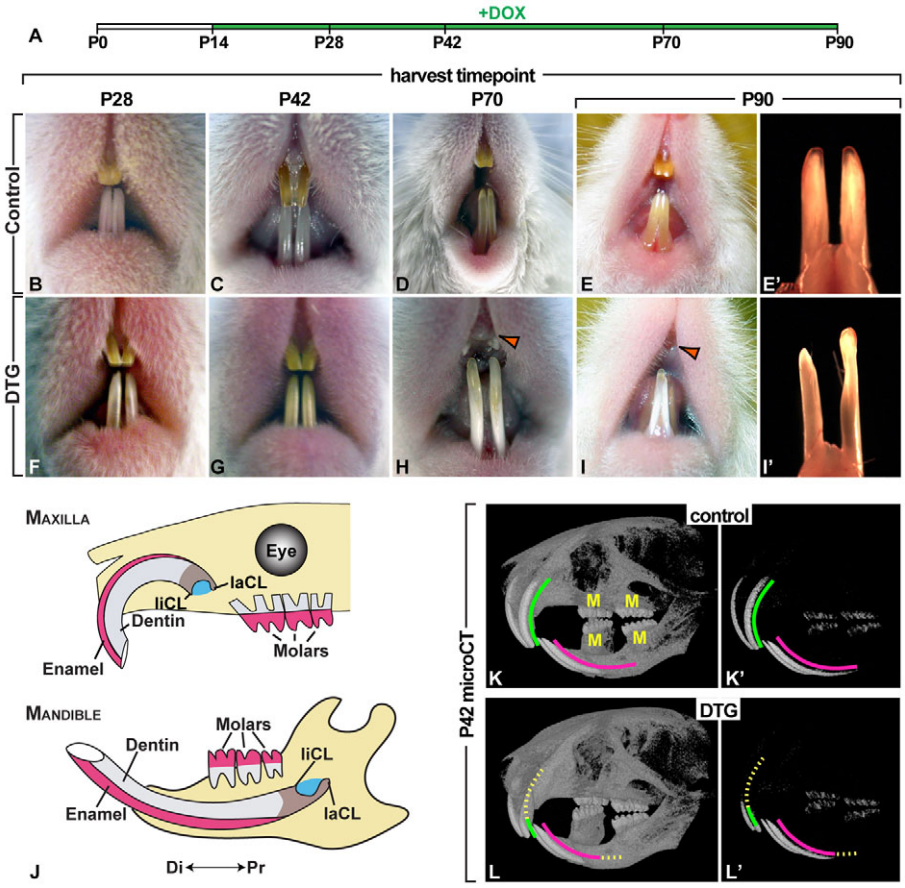

Fig. 3. Long-term attenuation of FGFR2b signaling in adult mice causes loss of maxillary incisors. (**A**) Timecourse for postnatal doxycycline treatment. (**B-I'**) Whole-mount images of control (B-E') and DTG (F-I') mice after 14 (B,F), 28 (C,G), 56 (D,H) and 76 (E,E',I,I') days of doxycycline treatment. (**J**) Diagram of the region analyzed by microCT. Blue represents mesenchyme and dark brown represents epithelium. (**K-L'**) MicroCT analysis showing enamel and bone (K,L) or enamel only (K',L'). Green line and pink line indicate enamel in the maxillary and mandibular incisor, respectively. Dotted yellow lines indicate the absence of enamel in the proximal regions of the DTG incisors. Di, distal; laCL, labial cervical loop; liCL, lingual cervical loop; M, molars; P, postnatal day; Pr, proximal.

was very low FGFR expression in the OEE. These results indicate that FGFR2b signaling is not required in the IEE to maintain FGFR2b expression.

Sonic hedgehog (*Shh*) expression marks T-A cells in the labial CL as well as pre-ameloblasts and ameloblasts (Bitgood and McMahon, 1995; Klein et al., 2008). Our results demonstrated a marked decrease in the expression level of *Shh* in DTG incisors (Fig. 2N) in comparison with control incisors (Fig. 2M). This suggests that loss of T-A cells is a result of downregulation of signaling by FGFR2b. To assess the status of mature ameloblasts in the DTG incisors, immunohistochemistry for amelogenin protein was performed. Our results indicated a significant decrease in amelogenin expression in DTG versus control incisors (data not shown), indicating that transient attenuation of FGFR2b signaling leads to abnormal amelogenesis.

Postnatal attenuation of FGFR2b signaling leads to loss of amelogenesis in maxillary incisors

To study the impact of FGFR2b downregulation on stem cell-driven growth of the adult incisor, postnatal DTG and control mice (n=3 each) were administered doxycycline-containing food for different lengths of time starting at P14 (Fig. 3). Hereafter, mice referred to as controls are either wild-type or single-transgenic animals from the same litter. At P14, mandibular and maxillary incisors in both control and DTG animals were of normal appearance (data not shown). After 14 or 28 days of treatment with doxycycline (at P28 and P42, respectively), both maxillary and mandibular incisors of DTG mice (Fig. 3F,G) were grossly indistinguishable from the corresponding control incisors (Fig. 3B,C). However, at P70 (following 56 days of doxycycline exposure), the maxillary incisors had almost disappeared and the mandibular incisors had grown excessively (Fig. 3H) compared with those of wild-type mice (Fig. 3D). The increased mandibular incisor length at this stage was most likely to be due to the absence of abrasion between the upper and lower incisors. At P90, the mandibular incisors in the mutants were also degraded compared with the controls (Fig. 3I,I′ versus 3E,E′), indicating severe enamel deposition defects in the mandible as well as the maxilla.

To assess the early phases of the enamel defects, we used microCT analysis to visualize enamel deposition along the entire proximal-distal axis of mandibular and maxillary incisors (Fig. 3J-L′). MicroCT was performed on control and DTG animals at P42, prior to the detection of obvious defects in the erupted portion of the tooth. In DTG mice, enamel was absent from the proximal regions of both maxillary and mandibular incisors (compare Fig. 3L,L′ with 3K,K′). Interestingly, the length of the enamel-free zone was greater in the maxillary incisor compared with the mandibular incisor (Fig. 3L′). The earlier onset of enamel deposition defects in the upper incisors might reflect different requirements for FGFR2b signaling in maxillary and mandibular incisors.

To understand the impact of the downregulation of FGFR2b on maxillary incisors at earlier time points, histological analyses were performed on incisors harvested and sectioned from mice treated for 2 (P28) or 4 (P42) weeks with doxycycline (see Fig. S2 in the supplementary material and Fig. 4). At P28, a time point at which no visible abnormalities could yet be detected in the external appearance of the maxillary incisors, serial coronal sections indicated a failure to develop new ameloblasts in proximal regions close to the CL (compare Fig. S2E with S2H in the supplementary material), whereas more mature ameloblasts could be detected in further distal

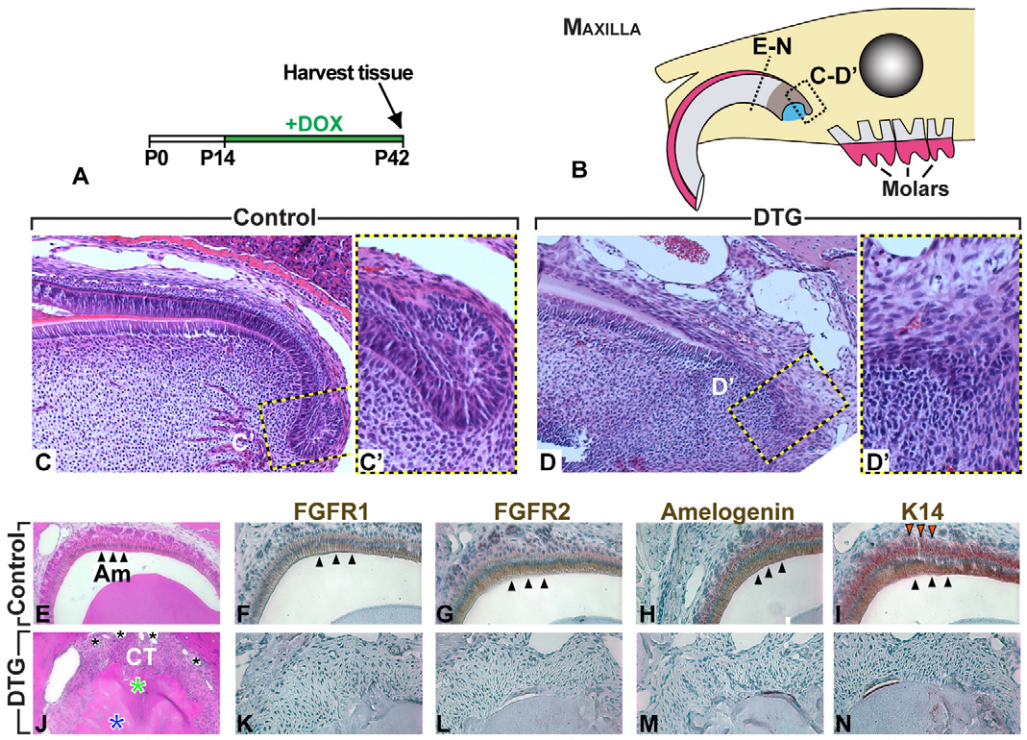

Fig. 4. Long-term postnatal reduction of FGFR2b signaling leads to defective amelogenesis in maxillary incisors. (**A**) Timecourse for doxycycline treatment. (**B**) Dotted lines in schematic indicate areas shown in sagittal sections in C-D′ and planes of coronal sections in E-N. Blue represents mesenchyme and dark brown represents epithelium. (**C-D′**,**E**,**J**) Hematoxylin and Eosin staining of maxillary incisor sections from control (C,C′,E) and DTG (D,D′,J) mice. C′ and D′ are magnifications of the areas indicated in C and D, respectively. Note defects in the dentin and enamel matrices (blue and green asterisks, respectively), ectopic blood vessels (small black asterisks), and connective tissue (CT) in the proximal incisor region (J) where ameloblasts are normally found in controls (E). (**F-I**,**K-N**) Immunostaining of coronal sections with antibodies against FGFR1 (F,K), FGFR2 (G,L), amelogenin (H,M) and K14 (L,N). Black arrowheads point to ameloblasts.

regions (compare Fig. S2C with S2F in the supplementary material). This observation suggests that FGFR2b signaling is not required for the maintenance of ameloblasts that have already formed but is, however, crucial for the formation of new ameloblasts.

Longer exposure to doxycycline treatment (28 days up to P42) led to more extensive and severe defects in the maxillary incisors of DTG mice (Fig. 4A-N). Sagittal sections at P42 demonstrated the presence of a rudimentary CL in DTG compared with controls (Fig. 4C-D′). In the posterior region adjacent to the CL, the space normally filled by enamel was instead filled by connective tissue and blood vessels in DTG mice (Fig. 4J). Also, the dentin was abnormally formed and attached to defective enamel (Fig. 4J). Corresponding maxillary incisor sections of doxycycline-fed control mice revealed normal ameloblasts, enamel and dentin (Fig. 4E). Cell proliferation analysis in the labial CL of DTG (n=3) and control (n=3) incisors treated for 4 weeks (see Fig. S2I in the supplementary material) demonstrated a significant decrease in cell proliferation in the mutant CL [5.7±2.0 (DTG) versus 22.6±6.9 (control) BrdU-positive cells/arbitrary unit of CL area; P=0.028].

To further investigate the impact of decreased FGFR2b signaling on the adult maxillary incisor, immunohistochemistry for FGFR proteins and incisor epithelial cell markers was carried out. Immunohistological staining in control mice demonstrated specific expression of FGFR1 (Fig. 4F), FGFR2 (Fig. 4G) and amelogenin (Fig. 4H) in the ameloblasts, and expression of K14 in both the ameloblasts and the OEE (Fig. 4I). None of these markers was expressed in DTG mice treated for 28 days (Fig. 4K-N), confirming the absence of ameloblasts. This result shows that although downregulation of FGFR2b signaling does not lead to a macroscopic phenotype at early stages of treatment, important changes are occurring at the cellular level and are observed in the region of the maxillary incisor near to the CL.

Postnatal downregulation of FGFR2b signaling leads to loss of amelogenesis and decrease of proliferation in the mandibular incisors

Next, our analysis focused on the role of FGFR2b in the growth of the mandibular incisor. It has previously been shown that *Nkx3.1*^{Cre}-mediated inactivation of *Fgfr2b* results in defective maxillary incisors and apparently normal mandibular incisors (Lin et al., 2009). In agreement with these results, we observed defective maxillary incisors in our DTG mice. However, our data also showed that enamel deposition in mandibular incisors was impaired after long-term treatment with doxycycline, albeit to a lesser degree compared with the phenotype displayed by the maxillary incisors. Histological analyses of control and mutant incisors were carried out at P42, after 28 days of doxycycline treatment (Fig. 5A-B″). Mandibular incisors of control mice had a normal enamel space covered by inner and outer enamel epithelia, well-formed CLs, and an odontoblast layer lining the dental pulp (Fig. 5A-A″). By contrast, the only recognizable epithelial structure in the treated DTG mandibular incisors was a rudimentary CL, whereas the odontoblast layer and dental pulp appeared grossly normal (Fig. 5B-B″; data not shown).

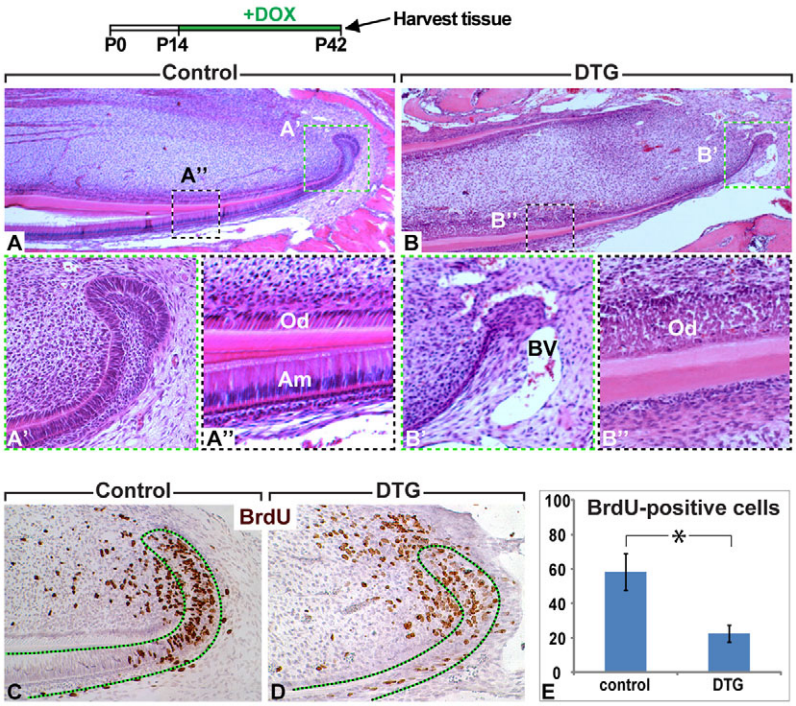

Fig. 5. Ameloblast formation and ameloblast progenitor proliferation are reduced after 4 weeks of postnatal FGFR2b signaling attenuation in mandibular incisors. (**A-B″**) Hematoxylin and Eosin staining of sagittal sections of mandibular incisors of control (A-A″) and DTG mice (B-B″) treated with doxycyline for 28 days (treatment timecourse shown at top). Dotted boxes in A and B indicate regions magnified in A′, A″, B′ and B″. Note the presence of ectopic blood vessels (BV) near the labial CL of DTG mice (B′). (**C**,**D**) BrdU staining on sagittal sections of control (C) and DTG (D) incisors. Dotted lines indicate the outline of the epithelium. (**E**) Number of BrdU-positive cells (mean ± s.e.m.) in the cervical loop of control and DTG mice. **P*<0.05. Am, ameloblasts; Od, odontoblasts.

To further examine the phenotype, immunostaining for ameloblast markers was carried out. Amelogenin, which is expressed by developing ameloblasts, was absent in mutant mice (data not shown). Similarly, K14 and E-cadherin, which are normally expressed in the IEE, OEE and SR of wild-type incisors, were absent from the IEE and OEE of DTG incisors (data not shown). These results confirm that ameloblast function is impaired in the mandibular incisors as a result of downregulation of the FGFR2b signaling pathway.

To study the impact of FGFR2b signaling on cell proliferation in the mandibular incisors, proliferation assays using BrdU incorporation were carried out in vivo and sagittal sections of 4-week-treated DTG and control mandibular incisors (n=3 for each) were examined. Analysis of BrdU incorporation indicated a drastic reduction in epithelial proliferation in the labial CL of DTG incisors (Fig. 5D) compared with controls (Fig. 5C). Quantification of the BrdU signal confirmed the reduction in proliferation [Fig. 5E; 22.5±5.0 (DTG) versus 58.2±10.7 (control) BrdU-positive cells/arbitrary unit of CL area; P=0.012]. Thus, attenuation of FGFR2b signaling resulted in a significant reduction of the proliferation rate of T-A ameloblast progenitors in the CL, which is likely to be the underlying cause of the enamel defects observed in the mandibular incisors.

Next, we investigated the effects of FGFR2 attenuation in the adult on gene expression in the proximal incisor. *Shh*, a known target of FGFR signaling during incisor development, was expressed at high levels in T-A cells, pre-ameloblasts and ameloblasts of control animals (Fig. 6A). In situ hybridization

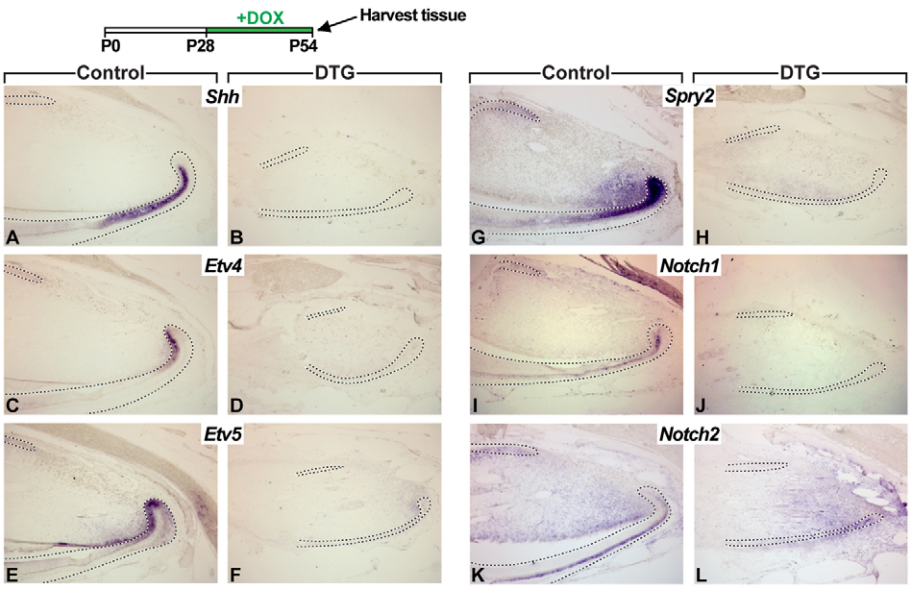

Fig. 6. Postnatal expression of dominant-negative FGFR2b causes downregulation of FGFR signaling target genes and changes in expression of dental epithelial markers. (**A-L**) In situ hybridization of sagittal sections of the proximal incisor of untreated controls (A,C,E,G,I,K) and doxycycline-treated DTG mice (B,D,F,H,J,L). Expression of *Shh* (A,B), *Etv4* (C,D), *Etv5* (E,F), *Spry2* (G,H) and *Notch1* (I,J) is markedly decreased in treated DTG mice relative to controls, whereas *Notch2* expression (K,L) appears to be maintained. Treatment timecourse is shown at the top.

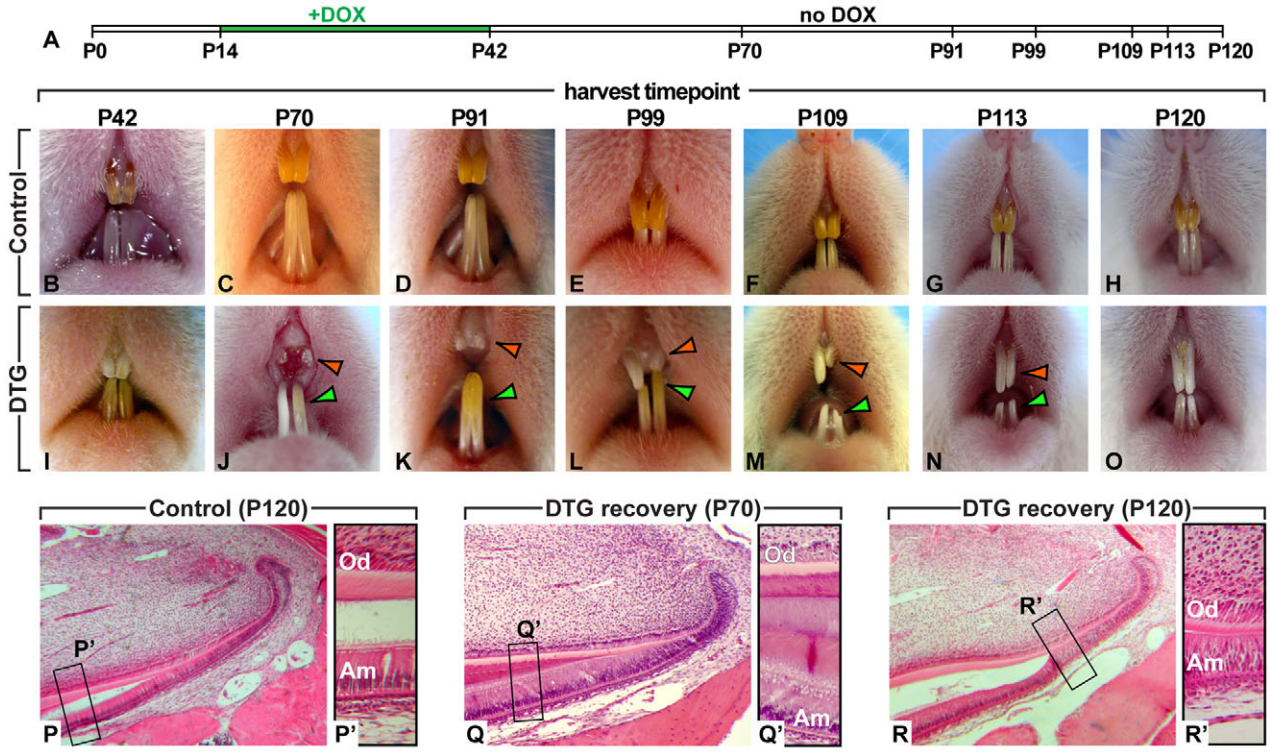

Fig. 7. Effects of 4-week attenuation of FGFR2b signaling in postnatal incisors are reversible. (**A**) Timecourse for postnatal doxycycline treatment followed by different lengths of doxycyline-free chases. (**B-O**) Whole-mount images of incisors of control (B-H) and DTG (I-O) mice after 4 weeks of doxycyline exposure and 0 (P42; B,I), 28 (P70; C,J), 49 (P91; D,K), 56 (P99; E,L), 66 (P109; F,M), 70 (P113; G,N) and 77 (P120; H,O) days doxycyline-free period before sacrifice. Red and green arrowheads point to abnormal maxillary and mandibular incisors, respectively. (**P-R'**) Hematoxylin and Eosin staining of proximal incisor sections of a control animal (P,P′) and DTG animals 28 days (P70; Q,Q′) and 77 days (P120; R,R′) post-doxycyline treatment. Boxes in P, Q and R indicate areas magnified in P′, Q′ and R′, respectively. Am, ameloblasts; Od, odontoblasts.

and quantitative RT-PCR demonstrated markedly reduced *Shh* expression in doxycycline-treated DTG mice (Fig. 6B and see Fig. S3 in the supplementary material). Furthermore, in control animals, the direct FGF targets *Etv4* and *Etv5* (O'Hagan and Hassell, 1998; Roehl and Nusslein-Volhard, 2001) were expressed at high levels in the T-A region and at lower levels in the proximal part of the labial CL, as well as in the mesenchyme adjacent to the region of T-A cells (Fig. 6C,E). By contrast, expression of both factors was barely detectable in doxycyline-treated DTG mice (Fig. 6D,F and see Fig. S3 in the supplementary material). *Spry2*, another target gene of FGFR signaling (Mason et al., 2006), was strongly expressed throughout the labial CL and in the mesenchyme adjacent to the IEE in control mice (Fig. 6G), whereas expression in treated DTG mice was greatly reduced (Fig. 6H and see Fig. S3 in the supplementary material). Downregulation of these factors confirms that the phenotype in the CLs of DTG mice was the result of decreased FGFR signaling. Furthermore, downregulation of direct target genes of FGF signaling in the labial CL indicates that the observed effects of FGFR2b attenuation on the labial CL were, in fact, direct. Interestingly, we detected a decrease in expression of *Notch1*, which is normally expressed in the SR and in stratum intermedium cells underlying the ameloblasts and their precursors (Harada et al., 1999), in treated DTG mice (compare Fig. 6I with 6J and see Fig. S3 in the supplementary material). By contrast, expression of *Notch2*, which is widely expressed in the incisor mesenchyme and marks the SR and OEE (Harada et al., 1999), was less affected, indicating that the rudimentary CL in treated DTG mice still contains at least one of these cell types.

Lastly, *Fgf10* expression had previously been examined only in the embryonic incisor. Because this gene encodes the ligand that is thought to be the principal FGF family member in the incisor mesenchyme, we set out to detect expression of this gene in the adult. Expression of the *Fgf10* mRNA transcript in adults is not always reliable; therefore, we generated a mouse line carrying a *lacZ* reporter allele within the *Fgf10* locus. We found intense expression throughout the mesenchyme, including in the mesenchyme between the CLs, the mesenchyme surrounding the labial and lingual CLs, and in the odontoblasts (see Fig. S4 in the supplementary material).

Although the above experiments pointed towards a mechanism in which FGF10 (secreted from the mesenchyme) signals through epithelial FGFR to drive proliferation in the T-A cells of the incisor, an important question is yet to be addressed: are stem cells also affected by decreased FGFR2b signaling in mandibular and maxillary incisors?

Defective maxillary and mandibular incisors resume normal growth upon cessation of doxycycline treatment

To elucidate the role of FGFR2b signaling in survival and/or proliferation of stem cells in the incisors, the reversibility of the incisor phenotype after long-term treatment of DTG mice with

doxycycline was tested (Fig. 7). Beginning at P14, DTG and control mice (n=4 per group) were given doxycycline-containing food for 4 weeks followed by different periods without doxycyline exposure (Fig. 7A). At P42, 28 days after beginning doxycycline treatment, no difference in gross incisor phenotype was observed (Fig. 7B,I). At P70, 28 days post-treatment, the tips of the maxillary incisors of DTG mice were broken and the mandibular incisors were transparent, suggestive of defective enamel deposition (Fig. 7J). Newly formed maxillary incisors began to grow by P99 (57 days post-treatment) (Fig. 7L). At this stage, the mandibular incisors lost their transparency. Between P99 and P109, the mandibular incisors broke as a result of contact with the newly formed maxillary incisors. By P120, the maxillary and mandibular incisors of DTG mice developed to a length that allowed for normal contact between maxillary and mandibular incisors (Fig. 7O). This experiment demonstrates that the incisor phenotype of DTG mice was reversible after reactivation of FGFR2b signaling.

To investigate the status of cells at the growth-initiating proximal end of the incisor, histological sections were prepared from control and DTG mandibular incisors at different time points following release of inhibition (Fig. 7P-R′). We found de novo formation of an enamel-forming epithelial layer in sagittal sections of the mandibular incisors, as well as a well-formed labial CL (Fig. 7Q-R′) of appearance similar to that of the controls (Fig. 7P,P′). Interestingly, reversal of the defects at the proximal end of the incisor was already observed at P70 (28 days post-treatment). At this time point, transparency of the lower incisor indicated abnormal enamel deposition on the erupted portion of the mandibular incisor.

In summary, adult DTG mice treated for 4 weeks (P14-42) lost the visible part of the maxillary incisors at P70, indicative of progressive ameloblast defects occurring at the level of the labial CL upon attenuation of FGFR2b signaling. However, re-growth of the maxillary incisors of DTG mice after the animals resumed a normal diet demonstrates that attenuation of FGFR2b signaling over the period tested here did not compromise the survival of the adult stem cells, as these cells still retained the ability to give rise to T-A progenitors that were able to properly differentiate to form enamel-producing ameloblasts. Thus, normalization of FGFR2b signaling in the DTG animal allows for resumption of proper ameloblast formation.

DISCUSSION

The ultimate goal of regenerative medicine is to harness the power of endogenous adult stem cells to allow for the recovery of affected organs after injury. Adult stem cells in many organs replace damaged cells by dividing asymmetrically, thus contributing to proliferating T-A cells that can mature into functional differentiated cells. Mouse incisors grow continuously during adult life and are, therefore, an ideal model to use for determining the signaling pathways that are essential for tooth regeneration. Our results, using a reversible and inducible system, demonstrate that attenuation of FGFR2b signaling does not compromise the survival of the stem cells located in the CL but instead leads to decreased proliferation of T-A progenitors. This, in turn, decreases de novo ameloblast formation, a defect visualized over time by insufficient enamel deposition in both the maxillary and mandibular incisors, with the upper incisors being more severely affected. Consistent with previous reports, we also found that inactivation of FGFR2b signaling during embryonic development (from E12.5-18.5) leads to the formation of a rudimentary CL in both the maxillary and mandibular incisors. The molecular and cellular basis for the differences observed between maxillary and mandibular incisors during development and adult life is still elusive, but our results suggest that attenuation of FGFR2b signaling is partially compensated in the mandibular CL by an alternative signaling pathway that remains to be identified.

Previously, it was reported that signaling mediated by FGF receptors is important for the maintenance and survival of the stem cells and T-A cells in the CL during development (Harada et al., 2002). More recently, experiments using the *Nkx3.1*^{Cre} driver line have shown that FGFR2b signaling controls the formation of the CL in the maxillary, but not in the mandibular, incisors during embryonic development (Lin et al., 2009). Our results differ from these, as we found that both the mandibular and maxillary incisors are affected during development, albeit with different severity. A possible explanation for this discrepancy is that the Cre driver line used in the previous study did not lead to high levels of recombination in the developing mandibular incisors.

In the labial CL of postnatal mice, the main ligands for FGFR2b are FGF3 and FGF10. Whereas *Fgf10* is expressed at high levels by the dental mesenchyme adjacent to the CL and IEE, both the CL and the IEE express FGF receptors *Fgfr1b* and *Fgfr2b*. *Fgf3* is predominantly expressed in the mesenchyme adjacent to the IEE (Harada et al., 1999). Differences between *Fgf3* and *Fgf10* expression domains are also observed during embryonic development at E16, when the CL is forming (Harada et al., 2002). The more restricted pattern of *Fgf3* expression suggests that *Fgf3* and *Fgf10* might be partially redundant during incisor development and homeostasis. These two ligands are thought to interact with crucial signaling pathways that control the fate of stem cells during incisor development and homeostasis (Harada et al., 1999; Wang et al., 2007). Because *Fgf10*-null animals die at birth from many defects, including lung agenesis (Harada et al., 2002), it has not been possible to study adult mice that completely lack *Fgf10* and *Fgf3*. Using our in vivo model of inducible expression of the dominant-negative FGFR2b, we achieved attenuation of both FGF3 and FGF10 signaling. Our data from postnatally treated mice reveal a more drastic phenotype than that reported in previous studies of *Fgf3*$^{-/-}$;*Fgf10*$^{+/-}$ adult incisors (Wang et al., 2007).

Recently, FGF3 and FGF10 in the mesenchyme have been shown to regulate *Fgf9* expression in the epithelium, which in turn regulates the expression of *Fgf3* and *Fgf10* in the mesenchyme (Klein et al., 2008). This FGF epithelial-mesenchymal signaling loop is regulated by members of the sprouty family, which act as intracellular inhibitors of the FGF signaling pathway (Mason et al., 2006). The function of sprouty proteins is to limit the formation of ameloblasts to the labial aspect of the incisors by inhibiting the establishment of a lingual FGF epithelial-mesenchymal signaling loop (Klein et al., 2008). The inactivation of sprouty genes corresponds to a gain of function of FGF signaling, providing additional support to our conclusions that FGFR2b signaling positively controls ameloblasts during homeostasis.

Our finding that maxillary and mandibular incisors regenerate after long-term attenuation of FGFR2b signaling indicates that, over the time period studied here, FGFR2b signaling does not control stem cell survival in the incisor. This is consistent with our gene expression data, which indicated that the population of cells in the labial CL receiving the highest levels of FGFR signaling was the T-A population. A similar conclusion was reached for FGFR2b signaling in epithelial stem cells in the adult mammary gland (Parsa et al., 2008). Interestingly, we did find that after very long-term attenuation of FGFR signaling (from P14-724), maxillary incisor growth after relief of inhibition was impaired (data not shown).

This finding, which we intend to pursue in future work, might be a result of the effects of FGF signaling on other cell populations that, in turn, signal back to the stem cells.

In the future, it will be important to further characterize incisor stem cells. In addition, it will be interesting to determine whether the regenerative capability of human teeth could be linked to FGFR2b signaling. Indeed, mutations in the FGF signaling pathway can severely affect the production of enamel in humans. Lacrimo-auriculo-dento-digital (LADD) syndrome has been linked to mutations in *Fgf10* and *Fgfr2b* (Rohmann et al., 2006), and patients with LADD exhibit enamel dysplasia (Hollister et al., 1974; Guven et al., 2008). Our mouse model is, therefore, in agreement with a crucial role for FGFR2b signaling in ameloblast formation in humans.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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