Glial origin of mesenchymal stem cells in a tooth model system

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Mesenchymal stem cells occupy niches in stromal tissues where they provide sources of cells for specialized mesenchymal derivatives during growth and repair1. The origins of mesenchymal stem cells have been the subject of considerable discussion, and current consensus holds that perivascular cells form mesenchymal stem cells in most tissues. The continuously growing mouse incisor tooth offers an excellent model to address the origin of mesenchymal stem cells. These stem cells dwell in a niche at the tooth apex where they produce a variety of differentiated derivatives. Cells constituting the tooth are mostly derived from two embryonic sources: neural crest ectomesenchyme and ectodermal epithelium2. It has been thought for decades that the dental mesenchymal stem cells3 giving rise to pulp cells and odontoblasts derive from neural crest cells after their migration in the early head and formation of ectomesenchyme4,5. Here we show that a significant population of mesenchymal stem cells during development, self-renewal and repair of a tooth are derived from peripheral nerve-associated glia. Glial cells generate multipotent mesenchymal stem cells that produce pulp cells and odontoblasts. By combining a clonal colour-coding technique6 with tracing of peripheral glia, we provide new insights into the dynamics of tooth organogenesis and growth.

Shortly after the dental placode is induced, nerves innervate intimately with the developing tooth7. To address whether glia-derived cells contribute to dental mesenchymal stem cells (MSCs) during tooth organogenesis, we used mouse strains allowing for permanent genetic labelling of multipotent8 Schwann cell precursors (SCPs) and Schwann cells. Proteolipid protein 1 (PLP1) and sex-determining region Y-box 10 (Sox10) are expressed in cranial neural crest, but after migration around embryonic days (E)9–10, they are retained in SCPs and not in mesenchyme4,5. SCPs at E11.5–12.5 express typical markers of Schwann cell lineage (Supplementary Information and Extended Data Fig. 1). PLP-CreERT2 and Sox10-CreERT2 mice9,10 were therefore used for lineage tracing of SCPs. We controlled the specificity of PLP1 expression at E12.5 (Fig. 1a, b) and confirmed SCP-selective recombination in PLP-CreERT2/R26YFP mice by injecting tamoxifen at E12.5. Twenty-four hours later, traced cells expressing yellow fluorescent protein (YFP+) were located along nerves (Fig. 1c, Supplementary Information and Extended Data Fig. 1m–p). CreERT2 protein was confined to Sox10+ SCPs (Supplementary Information and Extended Data Fig. 1q–r). After tracing for 36 h, mesenchymal YFP+/CreERT2+ cells appeared close to nerves at the tooth site (Supplementary Information and Extended Data Fig. 2a–d). Induction of recombination at E12.5 and harvesting at E15.5–17.5 resulted in numerous traced cells along peripheral nerves and inside developing incisors (Fig. 1d–f, Supplementary Information and Extended Data Fig. 3a–g). YFP+ cells formed streams towards the odontoblast layer in spatial coordination with YFP+ odontoblasts (Fig. 1d). This was independently confirmed in Sox10-CreERT2 embryos (Fig. 1i–k and Supplementary Information and Extended Data Fig. 3h–k). Sox10 is expressed in SCPs and not in mesenchyme at E12.5 and at E15.5 (Fig. 1g–h, Supplementary Information and Extended Data Figs 1a–c, g–k, q–s, 2e–o and 3l–n). YFP+ pulp cells and odontoblasts formed the same pattern as seen with PLP-CreERT2 tracing (Fig. 1i–k). Therefore, SCPs must contribute to pulp and odontoblasts since CreERT2 protein in Sox10-CreERT2 and PLP-CreERT2 embryos was confined to SCPs (Supplementary Information and Extended Data Figs 2a–d, m–p and 3c–k).

To examine if patches of SCP-derived odontoblasts and pulp cells have clonal structure, we crossed the PLP-CreERT2 mice to the R26RConfetti reporter strain that allows for colour-encoded identification of clones8. This experiment revealed an organized clonal relationship between SCPs, pulp cells and odontoblasts (Supplementary Information and Extended Data Fig. 4a–d) and demonstrated that SCP-derived single MSCs produce pulpal and odontoblast fates (Supplementary Information and Extended Data Fig. 4e). We next examined whether ectomesenchymederived MSCs generate the same fates and patterns as SCP-derived MSCs. We induced recombination in neural crest at E8.5 in PLP-CreERT2/R26RConfetti strain, before segregation of CreERT2 expression into glial lineage. Recombination in both nerve-associated cells and ectomesenchyme was confirmed at E9.5 (Fig. 2a). When embryos were analysed at E17.5, it became apparent that ectomesenchyme- and SCP-derived MSCs generate the same fates and patterns in pulp and odontoblast layer (Fig. 2b–f).

To address whether Schwann cells generate MSCs also in adult growing incisors, we first confirmed that all Sox10+ cells in the apical proliferative zone were nerve-associated (Supplementary Information and Extended Data Fig. 5) and expressed Schwann cell markers (Supplementary Information and Extended Data Fig. 6a–g). CreERT2 protein was found in the apex at nerve sites in Sox10-CreERT2 and PLP-CreERT2 teeth (Supplementary Information and Extended Data Fig. 6h–q). Additionally, expression of CreERT2 protein driven by the PLP-promoter was identified exclusively in a subpopulation of Sox10+ Schwann cells (Supplementary Information and Extended Data Fig. 6j–q). Next, we used PLP-CreERT2 and Sox10-CreERT2 animals to analyse the progeny in growing incisors. We injected tamoxifen at postnatal day 60–85 and analysed the teeth 2–3 days later. Small numbers of YFP+ cells appeared in numerous traced cells along peripheral nerves and inside developing incisors (Fig. 1d–f, Supplementary Information and Extended Data Fig. 3a–g). YFP+ cells formed streams towards the odontoblast layer in spatial coordination with YFP+ odontoblasts (Fig. 1d). This was independently confirmed in Sox10-CreERT2 embryos (Fig. 1i–k and Supplementary Information and Extended Data Fig. 3h–k). Sox10 is expressed in SCPs and not in mesenchyme at E12.5 and at E15.5 (Fig. 1g–h, Supplementary Information and Extended Data Figs 1a–c, g–k, q–s, 2e–o and 3l–n). YFP+ pulp cells and odontoblasts formed the same pattern as seen with PLP-CreERT2 tracing (Fig. 1i–k). Therefore, SCPs must contribute to pulp and odontoblasts since CreERT2 protein in Sox10-CreERT2 and PLP-CreERT2 embryos was confined to SCPs (Supplementary Information and Extended Data Figs 2a–d, m–p and 3c–k).

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Figure 1 | SCPs give rise to pulp cells and odontoblasts in the developing tooth. a, b, *In situ* hybridization with PLP1 riboprobe (a) on a section of developing mandibular incisor at E12.5 post-stained for TuJ1 (β-III-tubulin) " nerves (b). Arrows indicate identical locations. c, Mandible of PLP-CreERT2/R26YFP embryo traced from E12.5 to E13.5. See Extended Fig. 2 for details. d–f, Incisor (d) traced from E12.5 to E17.5, PLP-CreERT2/R26YFP embryo. e, Magnified region from d, f, Nerve and apical mesenchyme, confocal stack. In e, arrows indicate odontoblasts. g, h, *In situ* hybridization with Sox10 riboprobe (g) on an E15.5 mandible post-stained for TuJ1 " fibres (h). Arrows point at identical locations. i, j, Incisor at E17.5 (i) from Sox10-CreERT2/R26YFP embryo traced from E15.5. j, The area outlined in i; arrows indicate odontoblasts. k, E17.5 incisor from Sox10-CreERT2/R26YFP embryo traced from E15.5, confocal stack. Collagen IV labelling: blood vessels; YFP "; pulp cells and odontoblasts (arrows). a–k, Dotted line: enamel organ. Scale bars: 100 μm (a–d, g–i); 25 μm (e, f, j, k). CL1 and CL2 indicate labial and lingual aspects of cervical loop.

adjacent to nerves in the apical incisor (Fig. 3a–c, Supplementary Information and Extended Data Fig. 7a–e). After 5 days YFP " cells at the apex increased in numbers (Supplementary Information and Extended Data Fig. 7f–h). The fates of the progeny were examined at times exceeding the incisor self-renewal (from 30 days after tamoxifen injection). We found that Schwann cells give rise to dental MSCs producing pulp cells and odontoblasts in adult teeth (Fig. 3d–f, Supplementary Information and Extended Data Fig. 7i–k).

Tracing in PLP-CreERT2/R26RConfetti mice demonstrated that streams of traced cells were connected to clusters of odontoblasts originating from same recombination events in Schwann cells (Fig. 3g–r, Supplementary Information and Extended Data Fig. 7j–k). The streams appeared increasingly dispersed as they approached odontoblasts labelled by the same colour (Fig. 3m). Clonal streams of pulp cells and odontoblasts intermingled at borders with non-labelled or different-coloured cells (Fig. 3l–p). MSCs produced high numbers of offspring, only a part of which was localized proximally to the dental epithelium and later became pre-odontoblasts. The majority of the progeny acquired a pulpal fate and formed organized streams, with earlier cells progressively displaced distally. Consequently, pulp cells and odontoblasts from the same clone remained associated during growth. These data suggest that progenies of several clones compete for the limited space at the inner surface of the cervical loop and, thus, for the odontoblast fate and final contribution. Indeed, the proportion of odontoblasts within the progeny of a single stem cell varied widely (Fig. 3s). Accordingly, the proximity of an MSC to the cervical loop correlated with the amount of odontoblast-fated progeny varied widely (Fig. 3s). Correspondingly, pulp cells and odontoblasts from the same clone remained associated during growth. These data suggest that progenies of several clones compete for the limited space at the inner surface of the cervical loop and, thus, for the odontoblast fate and final contribution. Indeed, the proportion of odontoblasts within the progeny of a single stem cell varied widely (Fig. 3s). Accordingly, the proximity of an MSC to the cervical loop correlated with the amount of odontoblast-fated progeny and may thus regulate the balance between odontoblast and pulp fates within a single clone (Fig. 3i). Additionally, streams originating closer to the cervical loop contained more cells and connected to larger clusters of odontoblasts than more central streams (Fig. 3q, t–v, Supplementary Information and Extended Data Fig. 8). Lastly, we found no support for a hypothesis that odontoblasts and pulp cells are generated from different pools of MSCs.
To prove the importance of the innervation for tooth growth, we denervated incisors 24 h after tamoxifen injection in PLP-CreERT2/R26YFP mice. After 10 days we found almost no progeny in denervated teeth, while contralateral control teeth contained abundant YFP+ odontoblasts and pulp cells (Supplementary Information and Extended Data Fig. 8j–p). Thus, generation of a progeny from PLP+ cells is impaired without innervation.

We quantified the amount of Schwann-cell-derived progeny in PLP-CreERT2/R26YFP mice (Supplementary Information and Extended Data Figs 7i and 9a) and found that it varied from 8.23 ± 3.3% (single tamoxifen injection) to 47.28 ± 4.02% (multiple injections) (Supplementary Information and Extended Data Fig. 7m–o). Hence, in addition to Schwann cells and SCPs, there are other sources of dental MSCs, possibly pericytes, which generate odontoblasts in injured teeth13. We addressed whether pericytes could be derived from peripheral glia, using NG2 staining14 on sections from traced mice. However, NG2+ pericytes in teeth were never YFP+. Similar results were obtained in adult incisors (Supplementary Information and Extended Data Fig. 10). Thus, we exclude pericytes as an intermediate for the Schwann-cell- and SCP-derived pulp cells and odontoblasts.

Next we searched for stem cell markers in Schwann-cell-derived dental MSCs. Results from an array of methods strongly suggest that a population of Schwann-cell-derived dental MSCs are Thy1 (CD90)+ (Supplementary Information, Extended Data Fig. 9 and Supplementary Video 1).

Finally, we examined if Schwann-cell-derived cells produce regenerative dentine after trauma. We induced recombination in adult PLP-CreERT2/R26YFP mice, and allowed Schwann cells to generate progeny for 1 month. We then inflicted a confined damage to the tooth (Fig. 4a). Six days later, numerous traced cells were observed at the injury site, including odontoblast-like alizarin-red-positive cells adjacent to matrix (Fig. 4b–f). Such features were not seen in intact teeth (Fig. 4g–h).

To conclude, SCPs and Schwann cells contribute to development, growth and regeneration of teeth. The concept of glia-to-MSC transition expands the borders of the multipotency of SCPs15 and suggests that Schwann cells and SCPs are dormant neural-crest-like cells that can be recruited from nerves and contribute to peripheral tissues. On the basis of our results, Schwann cells and SCP might be the in vivo origin of neural-crest-derived multipotent stem cells identified in cultures of dissociated embryonic and adult tissues and designated as postmigratory cranial neural crest cells16 and skin-derived precursors17.
METHODS SUMMARY
We used PLP-CreERT2/R26YFP\(^{13}\), Sox10-CreERT2/R26YFP, Thy1-Cre/R26YFP\(^{4}\) and R26Confetti\(^{1}\) mouse strains. Immunohistochemistry and \textit{in situ} hybridization used standard protocols on frozen sections of embryos or adult teeth. Multispectral imaging used Zeiss LSM700 and Zeiss LSM780 confocal systems\(^{11}\).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Mouse strains and animal information. All animal work was permitted by the Ethical Committee on Animal Experiments (Stockholm North Committee) and conducted according to The Swedish Animal Agency’s Provisions and Guidelines for Animal Experimentation recommendations. Glia-specific genetic tracing mouse strains Plp-CreERT2/R26YFP, Sox10-CreERT2/R26YFP and Thy1-Cre/R26YFP
(Jackson Laboratory stock number 006143) were used in this study. R26Confetti mice were received from the laboratory of H. Clevers. To induce genetic recombination, pregnant females were injected intraperitoneally with tamoxifen (Sigma T5648) dissolved in corn oil (Sigma C8267). A range of tamoxifen concentrations (1.5–5 mg per animal) was used to gain different efficiency of genetic tracing. Mice were killed with isoflurane (Baxter KDG9623) overdose and perfused with PBS and then with 4% paraformaldehyde (Merck 818715) before collection of adult teeth. The tissue was additionally fixed for 3 h in 4% paraformaldehyde at 4°C on a rocking table, cryopreserved in 30% sucrose (VWR C27480) overnight at 4°C, embedded in OCT media (HistoLab 45830) and cut into 14 μm sections of the adult teeth. Antigen retrieval with Dako solution (Dako S1699) and quenching by peroxidase treatment (Merck 107209050) were done before application of primary antibodies to reduce background fluorescence. Primary antibodies were applied to the tissue and incubated at room temperature (21°C) overnight. Primary antibodies were CD31 (BD Phamingen 558745, 1:300), PECAM-1/CD31 (BD Phamingen 553370, 1:200), collagen IV (AbD Serotec 21501470, 1:500), NG2 (Millipore AB5320, 1:200), PGP9.5 (Cederlane CL95101, 1:500), SOX10 (Santa Cruz sc-17342, 1:500), CRE (Novagen 690050, 1:500), green fluorescent protein (GFP; Abcam ab6662, 1:500), ZF3 (generated by Developmental Studies Hybridoma Bank, provided by F. Lalemand, 1:100), BFABP (generated by T. Müller, 1:1000), Krox20 (Nordic Biosite AB, 1:500), Tuj1 (Promega G712A, 1:1000), S100P (DAKO AS110, 1:1000), p-c-fjun (Santa Cruz sc-822, 1:200), Ki67 (Thermo Scientific RM-9106-51, 1:1000), GFAP (Abcam ab2760, 1:500), Thy1 (Abcam ab1035, 1:500), P75 (Promega G23A, 1:1000), MBP (Abcam ab7349, 1:200 and Ab963 (R&D AF6418, 1:500). Additional secondary antibodies were Alexa secondary antibodies (Invitrogen, 1:800–1:1000) were used and slides were mounted with glycerol mounting media (Merck 1040942500). Additional stainings were done with 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 15–100 min at room temperature and Alizarin red (Sigma A5533, 2% in distilled H2O applied for 30 s to 3 min).

In situ hybridization on sections was done as previously described. Plp1 probe corresponded to the open reading frame of PLP1 protein with National Center for Biotechnology Information (NCBI) accession number NM_011123. Sox10 probe was a gift from T. Müller. Embryo heads were fixed in 4% paraformaldehyde for 2 h and then rinsed in 30% sucrose overnight at 4°C. Subsequently, the samples were immersed in OCT and frozen at −20°C until sectioning. Images were taken with Carl Zeiss Axioplan 2 light microscope.

Thyl antisense probes were generated by 2.3 kilobase complementary DNA fragment sequences cloned into pCMV-SPORT6 vector and transcribed in the presence of digoxigenin-labelled UTP, using Kpn1/T7 DIG RNA labelling kit (Roche). Whole-mount digoxigenin-labelled in situ hybridization was performed on 5-day postnatal CD1 pups. Incisor samples after whole-mount in situ hybridization were embedded in 1% low-melting-point agarose, dehydrated in methanol and cleared in BABB (two parts benzyl benzoate to one part benzyl alcohol) alcohol before optical projection tomography scanning. Optical projection tomography scanning was performed using a Biopontics 3001 OPT scanner (Biopontics). Reconstructed images were processed using 3DChimera software and further assessed using a Biopontics viewer version 2.0 (Biopontics).

Tooth damage. Tamoxifen (5 mg) was administered once a day for two consecutive days to the adult Plp-CreERT2/R26YFP animals before a tracing period of 4 weeks, which allowed tracing of both the glia-derived progenitor cells and their progeny forming streams in the dental pulp. Traced mice were anaesthetized by intraperitoneal injection of a combinitinal drug consisting of 75 mg per kg ketamine and 1 mg per kg medetomidinhydrochlorid prepared in sterile water for injection. Then 0.08 mg per kg Tamgesic dissolved in sterile saline solution was injected intramuscularly. When anaesthetized and sedated, the eyes of the animals were covered with eye gel (Oculement simplex APL, 336164). The operation site was sterilized with ethanol, and a 15 mm incision of the facial skin over the right masseter muscle was made. The mandibular bone surface was then exposed through a careful blunt dissection of the muscle. Care was taken not to damage the superficial muscle branches of the facial (VII) nerve. The thin bone layer covering the mandibular canal cranial to the incisor apex was carefully removed and the inferior alveolar nerve was exposed and transected with microscissors. Finally, muscle and cutaneous tissue were sutured. To awaken the animals, Anti-sedanVet (OrionPharma 471953) was given by subcutaneous injection. Wet food was administered to the animals during the survival period. The mice were monitored twice a day and given Tamgesic for pain relief every 12 h for the first 3 days and then once a day thereafter until they were fully recovered. Subsequently, they were continuously monitored. After 10 days, 4% paraformaldehyde perfusion was performed, and both mandibular incisors were then dissected out and processed for microscopy.

Flow cytometry. Dental pulps were obtained from the incisors of Plp-CreERT2/R26YFP animals, where the genetic tracing was induced during adulthood for a period of 4 weeks or longer. For each experiment, eight dental pulps were incubated by shaking at 225 r.p.m. in a mixture of collagenase/dispose (2.5 mg ml⁻¹; Roche 110971 13001) dissolved in 1× TrypLE Express (Gibco) for 1 h at 37°C. After the enzymatic dissociation, cells were sorted using cell strainers and washed in PBS. For subsequent staining, cells were used either live or fixed in cold methanol (45 min on ice followed by wash with PBS) depending on the applied antibody. Cell suspension was first incubated with primary antibodies in PBS at a concentration of 4 µg ml⁻¹ (anti-Thy1, anti-Ki67, anti-GFP) for 45–60 min on ice and washed with PBS afterwards. Secondary antibodies diluted 1:1000 in PBS (Alexa Fluor 405, 488 and 647; Life Technologies) were applied to the cell suspension for 45 min on ice. Protected from light and then washed with PBS. Flow-cytometry analysis was performed using FACSCanTofl and BD FACS DIVA 6.1.3 software. Each experiment with a given staining combination was performed in triplicate and controlled by using unstained sample and the corresponding isotypic IgG control (BD Biosciences).

BrdU and EdU incorporation analysis. BrdU was injected once or every day for 3 weeks at a concentration of 200 µg per g body weight. Mice were then killed after different periods (8 h, 24 h, 32 days, 64 days) and the tissue was sampled. Immunostaining and detection of BrdU was done according to a standard protocol (Abcam BrdU Immunohistochemistry Kit ab125306). Genetic tracing with label-retaining assay (Supplementary Fig. 13e–j): PLP-CreERT2/R26YFP mice were injected with 5 mg tamoxifen twice within two sequential days. After 7 months of genetic tracing, five injections of EdU (65 µg per g body weight, every 48 h) were performed. Seventy-one days after the last EdU-injection, the animals were killed and tissue samples fixed in 4% paraformaldehyde in PBS. Flow cytometry was performed using a BD FACSAria II cell sorter. EdU injections (3.3 µg per g body weight) for 3 weeks and washed out for another 3 weeks before collection. The incisor pulp cells were freshly collected and EdU staining for flow cytometry was performed according to the manual (Invitrogen).

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Thy1-FITC antibody (Ebioscience) was incubated with cells for 10 min at room temperature before cell fixation and EdU detection. A BD Fortessa cell analyser (BD Biosciences) was used with FACSDIVA software for acquisition and Flowjo software for analysis. EdU-Alexa647, Thy1-FITC and DAPI were detected with laser 633 nm, 488 nm and 351 nm excitation and with emission filter 670/30 nm, 530/30 nm and 450/50 nm respectively.

**Cell counting and statistics.** Statistical data are represented as mean ± s.e.m. Unpaired and paired versions of Student’s *t*-test were used to calculate the statistics (*P* value). Pearson’s product-moment correlation coefficient (*r*) was calculated to investigate the association of variables in Fig. 3t–v (*n* = 29 for Fig. 3t–u and *n* = 27 for Fig. 3v). Every value corresponding to a dot refers to a single clone; in total, clones were analysed from 11 different animals. Linear regression was used to build an approximation line in Fig. 3t. To analyse the position of dental MSCs in relation to the cervical loop (Fig. 3t–u), multiple sequential sections were analysed. Generally, we devoted several (always more than three) animals to every experiment to accomplish at least a biological triplicate. This was valid for all non-quantitative analyses including work done on sections or in a whole mount. For the genetic tracing experiments reported as graphical panels, at least six embryos derived from at least two females were analysed; in most cases 15–20 embryos were used before conclusions and supporting graphics were generated. During our study more than 100 genetically traced animals of different strains were analysed before concluding final results. The animals were selected and distributed into groups in all experiments randomly. The control for the denervation experiment was an internal control coming from the same animal: the non-operated contralateral side (biological (number of individual animals) *n* = 5, while technical (number of sections analysed) *n* = 15). To quantify the contribution from Schwann-cell-derived MSCs, we analysed three animals per condition counting three sections in every animal (technical *n* = 9). YFP<sup>+</sup> cells and DAPI<sup>+</sup> cell nuclei inside the tooth were identified on confocal images, segmented in IMARIS software and counted in a semi-automated way.

**Microscopy and imaging.** Confocal microscopy used Zeiss LSM700 CLSM and Zeiss LSM780 CLSM instruments. Image processing and analysis used ZEN2010 and Imaris software. The settings for the imaging of Confetti fluorescent proteins were previously described<sup>6</sup>. For Fig. 2a the imaging of the confocal stack was done with a Zeiss LSM780 CLSM, Plan-Apochromat ×10/0.45 M27 Zeiss air objective, 23 optical slices of 12 μm each with the *z*-axis shift of 12 μm for every step. For Supplementary Fig. 1a–c the whole-mount staining, imaging and reconstruction of an embryo were done with instruments, objectives and software according to a published protocol<sup>11</sup>.
Extended Data Figure 1 | Expression of SCP markers and genetic tracing in nerves of E11.5–13.5 embryos. a–c, Whole-mount immunohistochemistry performed on an E11.5 mouse embryo with antibodies against 155 kDa neurofilaments (2H3), MITF and Sox10. Note that Sox10

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cells are localized at the nerves in maxillary and mandibular regions. d–f, Whole-mount immunohistochemistry performed on an E11.5 mouse embryo with antibodies against 155 kDa neurofilaments (2H3) and BFABP. d–e, Arrows point at posterior ventral spinal nerves hosting immature embryonic glial cells that lack BFABP expression. f, Magnified area outlined in d by white rectangle. Roman digits show corresponding numbers of cranial ganglia. Dotted line follows the contour of an embryo. g–l, Sagittal section through E12.5 mandible stained for Sox10 (g–l), Tuj1 and BFABP (g), GFAP (h) and p75 (i). j–l, ErbB3 is expressed in all Sox10

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nerve-adjacent SCPs. k–l, Magnified area outlined by white rectangle in j. Dotted line indicates epithelial organ. m–o2, Sagittal section through E13.5 mandible, sequential single plane optical slices 2 μm in z-axis. m1–m2, Magnified area outlined in m; n1–n2, magnified area from n; o1–o2, respectively, show an area from o. p1–p2, Region on a section shown in m–o, maximum intensity projection image. p1, p2, Magnified area that is outlined by white rectangle in p. m–p, White dotted line indicates incisor placode. q–t, Expression of CreERT2, Sox10 and Tuj1 in a mandible traced from E12.5 to E13.5 in PLP-CreERT2 embryo. Note the full co-localization of Sox10 and CreERT2. Sox10

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/CreERT2 cells are adjacent to the innervation. Scale bars, 100 μm (a–c, g–i); 500 μm (d–f); 50 μm (j–l); 50 μm (m–t).
Extended Data Figure 2 | Expression of Sox10, YFP and CreERT2 in a genetically traced mouse embryonic head. **a–d,** Expression of CreERT2 protein in a 36 h genetically traced PLP-CreERT2/R26YFP embryo at E13.5. **c, d,** Magnified areas outlined in **a** and **b.** Open arrows point at CreERT2/YFP cells attached to the nerve whereas filled arrow points at CreERT2/YFP cell proximal to the nerve. **e, f,** In situ hybridization with Sox10 riboprobe (e) on a section of a mouse embryonic E12.5 head post-stained with TuJ1 (β-III-tubulin, neuronal marker) antibody (f). **g–h,** In situ with Sox10 probe on a section of E12.5 developing mandible (g) with incisor tooth bud (outlined by dotted line) post-stained with TuJ1 antibody (h). **i–j,** Sox10 and PGP9.5 (marker for neurites) are visualized by immunohistochemistry on a section of an embryonic mandible at E12.5. Dotted line outlines incisor bud. **k–l,** Sections of developing mandible with incisor (k) and molar (l) buds from a Sox10-CreERT2/R26YFP embryo genetically traced from E12.5 to E13.5 and stained with antibody against the neuronal marker PGP9.5. Arrow in l points at developing cartilage. **m–p,** Expression of CreERT2 protein in a 24 h genetically traced Sox10-CreERT2/R26YFP embryo at E13.5. The developing Meckel’s cartilage is outlined by the red dotted circle in m and o. **n, p,** Magnified areas outlined in m and o. White dotted line shows the borders of tooth bud. Scale bars, 200 μm (**e–h**); 100 μm (**i, m, o**); 50 μm (**a, b, j–l, n–p**); 10 μm (**c–d**).
Extended Data Figure 3 | Expression of Sox10 and CreERT2 at intermediate stages of incisor development. a–g, Incisor traced from E12.5 to E15.5 in PLP-CreERT2/R26YFP animals. Enamel organ is outlined by dotted line.
a, b, YFP cells in the nerve and in the pulp are shown together with Ki67 staining. c–g, Note that the expression of CreERT2 is confined to nerve sites only, as shown in low (c, d) and high (e–g) magnification images of developing incisor. e–g, Area from c, d shown at high magnification. YFP/CreERT2 cells are indicated by arrows in e–g. Note the presence of CreERT2 /YFP proximally to the nerve and in the apical mesenchyme.

h–k, Incisor traced from E15.5 to E16.5 in Sox10-CreERT2/R26YFP animals. Panel i represents a magnified area from h, j, k. Magnified area outlined by white rectangle in i. Arrows in j and k point at CreERT2 /YFP cells in the apical mesenchyme; note that the expression of CreERT2 is confined to the nerve sites only.

l–m, Developing incisor at E15.5 stained with antibodies against Sox10 to show SCPs, Tuj1 to visualize nerves and Col IV to outline the position of vessels. Note that all Sox10 cells are nerve adjacent. n, Schematic drawing showing the position of Sox10 cells during early bell stage of tooth development. Scale bars, 50 μm (a–d, h, i, l, m); 25 μm (e–g, j, k). CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.
Extended Data Figure 4 | Sub-optimal genetic recombination highlights clonal relationships between pulp cells and odontoblasts during tooth organogenesis. 

a–c, Consecutive sections through the incisor from PLP-CreERT2/R26Confetti mouse embryo traced from E12.5 to E17.5 (sub-optimal recombination). Note the pulp and odontoblasts progenies of CFP<sup>+</sup> SCPs (white arrows in a and b). 

c, Section throughout the same tooth where cyan arrows point at columnar CFP<sup>+</sup> odontoblasts. 

d, Section through another clonally traced (sub-optimal recombination) incisor. Note the presence of YFP<sup>+</sup> clone in the pulp. Arrow points at a single YFP<sup>+</sup> cell positioned at the innervation site. 

e, Reconstructed lineage tree of the neural-crest-derived compartment in the tooth. 

a–d, Scale bars, 100 μm. CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.
Extended Data Figure 5 | Distribution of Sox10+ cells in adult incisor.

a, Localization of Sox10+ cells and Tuj1+ nerve fibres in the pulp of adult incisor. Note that apical mesenchyme between the aspects of a cervical loop contains a population of nerve-adjacent Sox10+ cells while the distal pulp harbours an additional population of Sox10+ cells (outlined by a yellow dotted line) that are not adjacent to the nerves. b–e, Magnified areas from a where they are outlined by numbered white rectangles. b, Magnified image of apical mesenchyme and cervical loop area with nerve-adjacent Sox10+ cells pointed out by arrows. c, Region in the distal pulp showing scattered Sox10+ cells. Note that these cells are not in contact with Tuj1+ fibres. d, Cervical loop area including a region of proximal pulp and apical mesenchyme. e, Neural bundle in the apical mesenchyme with nerve-adjacent Sox10+ cells pointed out by arrows (projection of a stack). f–k, Localization of Sox10+ cells in relation to the proliferative growth zone outlined by the expression of Ki67. g–h, Magnified cervical loop area outlined in f by white rectangle 1. Arrows point at detected Sox10+ cells. i–j, Magnified area shown in f by white rectangle 2. Arrows point at Ki67+ cells in the distal pulp. Note that Ki67 does not label the distal population of Sox10+ cells outlined by yellow dotted line in f. k, Schematic drawing showing two separated populations of Sox10+ cells in the pulp: one population is represented by nerve-adjacent cells and located in proliferative apical mesenchyme while another population is scattered in distal pulp at a significant distance from a cervical loop. Scale bars, 100 μm (a, b, f); 50 μm (c–e, g–j). CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively. White dotted line shows enamel organ epithelium.
Extended Data Figure 6 | Expression of Schwann cell markers and CreERT2 in adult incisor. a, Schematic drawing showing the position of a focus area (red frame) selected for demonstration of Schwann cell markers in b–g. Schwann cell markers are expressed in Sox10− nerve-adjacent cells located in apical mesenchyme: S100β and P0 in b, P0 and p-c-Jun in c, p75 and Krox20 in d, MBP in e and GFAP and P0 in f–g. Expression of CreERT2 under the control of Sox10− (o, p) and PLP1− (q–u) promoters in adult incisor. h, i, Note that CreERT2 protein is found in cells adjacent to the Tuj1− nerve fibres in the apical mesenchyme of 30-day traced Sox10-CreERT2/R26YFP animals. j–q, CreERT2 protein is detected in the nuclei of Sox10− cells (58 ± 7.9% of nerve-adjacent Sox10− cells are CreERT2−, n = 5) in the apical mesenchyme of 20-h traced PLP-CreERT2/R26YFP animals. i–n, Magnified area outlined in j, k. Arrows point at CreERT2− nuclei. o, p, Expression of CreERT2 is not detected in a population of Sox10− non-glial cells in distal pulp. q, Expression of CreERT2 is not found in a cervical loop area. Scale bars, 50 μm. CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively. Dotted line shows enamel organ epithelium.
Extended Data Figure 7 | Short and long tracing intervals reveal YFP<sup>+</sup> cells at initial positions and uncover the later contribution of Schwann-cell-derived cells to the growing incisor.  

**a–e.** Section through adult incisor traced for 2 days from a Sox10-CreERT2/R26YFP mouse stained for collagen IV and Tuj1 to visualize blood vessels and nerves, respectively.  

**b, c.** Magnified area outlined by rectangle 1 in a. Arrows point at YFP<sup>+</sup> cells adjacent to the innervation of a vascular bundle between cervical loops.  

**d, e.** YFP<sup>+</sup> cells (arrows) also appear adjacent to the nerve fibres of vascular bundle distally from cervical loop (position is outlined by rectangle 2 in a). Images represent maximum intensity projections of confocal stacks.  

**b, e.** Magnification area outlined by rectangle 1 in a. Arrows point at YFP<sup>+</sup> cells adjacent to the innervation of a vascular bundle between cervical loops.  

**d, e.** YFP<sup>+</sup> cells (arrows) also appear adjacent to the nerve fibres of vascular bundle distally from cervical loop (position is outlined by rectangle 2 in a). Images represent maximum intensity projections of confocal stacks.  

**i–j.** Ki67 labelling of a section of an incisor traced for 6 months from an adult PLP-CreERT2/R26YFP mouse, single tamoxifen injection at sub-optimal concentration.  

**i.** Arrows point at a cluster of YFP<sup>+</sup> odontoblasts (magnified in inset). Note the presence of YFP<sup>+</sup> streams of cells in the pulp after 6 months of genetic tracing.  

**j, k.** YFP<sup>+</sup> genetically traced pulp cells are positive for Ki67 only in proximity to the cervical loop.  

**k.** Magnified area outlined by white rectangle in j. Note the absence of Ki67<sup>+</sup> cells in the odontoblast layer and in pulp cells at a distance from the cervical loop (CL).  

**l, Scheme of genetic tracing experiments involving single and serial tamoxifen injections. m, n.** Segmentation of odontoblast and pulp cell nuclei in the adult genetically traced incisor (m) injected eight times with subsequent identification of YFP<sup>+</sup> nuclei (n) for semi-automated counting in IMARIS. Magnified areas outlined by rectangles are shown in the insets; m.p.i., months post-injection.  

**i–n.** CL1 is a labial cervical loop; dotted line outlines enamel epithelium with adjacent hard matrix.  

**o.** Contribution of Schwann-cell-derived cells to the incisors from single and multiple injected PLP-CreERT2/R26YFP animals (n = 3 for each type of experiment). For the quantification of contribution to the odontoblast lineage, only labial odontoblasts were analysed.  

**a–k, Scale bars, 100 μm (a, f–j); 50 μm (b–e, k); 100 μm (i–j); 25 μm (k).**  

CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.
Extended Data Figure 8 | Clonal analysis reveals diversity of progeny originating from different Schwann-cell-derived MSCs that are nerve dependent. a–f, Consecutive sections of an incisor traced for 1.5 months from a PLP-CreERT2/R26Confetti mouse (sub-optimal recombination). 

b, d, f, Magnified areas from a, c and e respectively. Note numerous dispersed RFP^+ pulp cells in a–d and large amounts of adjacent RFP^+ odontoblasts in c–f. g–i. Another example of an incisor traced for 1.5 months (g) from a PLP-CreERT2/R26Confetti mouse also stained for PGP9.5 to visualize nerve fibres. h, i. Magnified areas from g. Note the narrow stream of RFP^+ cells in the middle of the dental pulp in g and h, j, k. Contralateral control (j) and denervated (k) incisor teeth 10 days after inferior alveolar nerve transection surgery and 11 days after initiation of genetic tracing by single tamoxifen injection (PLP-CreERT2/R26YFP animals). Arrows in j point at abundant clusters of YFP^+ odontoblasts and pulp cells. White dotted line indicates enamel organ and hard matrix. 1–m, Wallerian degeneration within the distal stump of the inferior alveolar nerve after the surgery, an area outlined by red rectangle in k. n. Control nerve. o. Quantification of YFP^+ progeny in denervated and contralateral control teeth 10 days after surgery (paired t-test P < 0.0001, mean difference 86.6, 95% confidence interval 83.58–89.63; n = 5). p. Quantification of distances between a cervical loop and most distal YFP^+ progeny in control (1,479 ± 246.5 μm) and denervated (282.4 ± 71.52 μm) teeth 10 days after the surgery (unpaired t-test P = 0.0016, n = 5). a–i. Scale bars, 100 μm (a, c, e, g); 25 μm (b, d, f, h, i); 100 μm (j, k); 50 μm (l–n). CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.
Extended Data Figure 9 | Schwann-cell-derived MSCs express stem cell marker Thy1. a–c, Flow cytometry analysis of cell populations from dissociated incisor pulp cells. Incisors were isolated from two PLP-CreERT2/R26YFP animals that were traced for 5 months after a single tamoxifen injection (5 mg). a, Dot plots of isotypic control and immunostaining of non-fixed dissociated pulp cells on the basis of expression of YFP. A region, P1, has been drawn around the YFP<sup>+</sup> cells (shown in black). b, Dot plots of isotypic control and immunostaining of non-fixed dissociated pulp cells on the basis of Thy1 expression. A region, P2, has been drawn around the Thy1<sup>+</sup> cells (shown in blue). c, Dot plots of selected sub-populations on the basis of expression of proliferative marker Ki67 and Thy1, staining on fixed cells. YFP<sup>+</sup> cells are shown in black. Right panel shows population of YFP<sup>+</sup> cells only. A region, P3, has been drawn around Thy1<sup>-</sup>/Ki67<sup>+</sup> cells. P4 has been drawn around Thy1<sup>+</sup>/Ki67<sup>-</sup> cells. d, d<sub>1</sub>, Immunostaining for Thy1 and Tuj1 in an adult incisor. d<sub>1</sub> Magnified area outlined by white rectangle in d. e–f, Ki67, Sox10 and Thy1 in dental MSC niche between labial and lingual aspects of a cervical loop. Arrows point at Sox10<sup>+</sup> nuclei of Schwann cells. Note that some Schwann cells are Ki67<sup>-</sup>. Inset in e shows only the Thy1 staining channel from area outlined by white rectangle. f, Magnified area outlined by white rectangle. g–h, Incisor from a PLP-CreERT2/R26YFP mouse genetically traced for 8 months. Note Thy1<sup>+</sup>/YFP<sup>+</sup> cells located proximally to the cervical loop CL1 in the low-magnification image (g) and indicated by arrows in magnified images (g<sub>1</sub>–h). Magnified area is highlighted by white rectangle in g, i, j, Genetically traced incisor from a Thy1-Cre/R26YFP mouse. Note abundant progeny in the pulp (i, j) and odontoblast layer specifically shown in inset in j, k–m<sub>2</sub>, Genetically traced incisor from a Thy1-Cre/R26Confetti mouse. Note streams of YFP<sup>+</sup> cells in the pulp (k–l<sub>1</sub>) and a few odontoblasts (m<sub>2</sub>). Magnified area outlined by white rectangle in k. Dotted line marks enamel epithelium and mineralized matrix. n–q, BrdU incorporation analysis of fast and slowly cycling cells in the incisor. r, s, EdU<sup>-</sup> slowly cycling cells 71 days after the last EdU injection in Thy1-stained and genetically traced incisor (9 months after last tamoxifen injection). t, u<sub>1</sub>, Magnified area from r and s outlined by white rectangle. Arrows indicate Thy1<sup>+</sup>/EdU<sup>+</sup> cells. x–z, Flow cytometry analysis of EdU-retaining and Thy1<sup>+</sup> cells. x, Dot plot showing the gating for Thy1<sup>+</sup> cell. y, Dot plot showing the gating of EdU<sup>+</sup> cells among total DAPI<sup>+</sup> population. Note that EdU<sup>+</sup> cells represent 4.3% of total cell numbers. z, Dot plot of subpopulations on the basis of expression of Thy1 and incorporation of EdU. EdU<sup>+</sup>/Thy1<sup>+</sup> cells are in the upper right square, constituting 1.34% of total population. Scale bars, 100 μm (d, k, l); 50 μm (g, j); 100 μm (n–s); 25 μm (t<sub>1</sub>–w<sub>2</sub>). CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.
Extended Data Figure 10 | Populations of pericytes and glia-derived cells do not overlap in the tooth. a–e, Sox10 and NG2 immunohistochemistry on a section of mandibular incisor from a PLP-CreERT2/R26YFP embryo genetically traced from E8.5 to E17.5. a, Dotted line outlines enamel organ. b, c, and d, e, Magnified areas from a outlined by white rectangles. Arrows in d, e show NG2+/YFP+ pericytes. f, g, YFP+/NG2+ pericytes (arrows) in the forebrain of an E8.5 to E17.5 genetically traced PLP-CreERT2/R26YFP embryo.

h, Immunohistochemistry with Sox10 and NG2 antibodies on a section of mandibular incisor traced for 30 days in a PLP-CreERT2/R26YFP adult mouse. Note the stream of YFP+/NG2+ cells in the pulp of the incisor and numerous YFP+/NG2+ pericytes on the same section. a–h, Scale bars, 50 μm (a); 25 μm (b–h). CL1 and CL2 are the labial and lingual aspects of the cervical loop respectively.