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Fate map of the dental mesenchyme: Dynamic development of the dental papilla and follicle

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ABSTRACT

At the bud stage of tooth development the neural crest derived mesenchyme condenses around the dental epithelium. As the tooth germ develops and proceeds to the cap stage, the epithelial cervical loops grow and appear to wrap around the condensed mesenchyme, enclosing the cells of the forming dental papilla. We have fate mapped the dental mesenchyme, using *in vitro* tissue culture combined with vital cell labelling and tissue grafting, and show that the dental mesenchyme is a much more dynamic population then previously suggested. At the bud stage the mesenchymal cells adjacent to the tip of the bud form both the dental papilla and dental follicle. At the early cap stage a small population of highly proliferative mesenchymal cells in close proximity to the inner dental epithelium and primary enamel knot provide the major contribution to the dental papilla. These cells are located between the cervical loops, within a region we have called the body of the enamel organ, and proliferate in concert with the epithelium to create the dental papilla. The condensed dental mesenchymal cells that are not located between the body of the enamel organ, and therefore are at a distance from the primary enamel knot, contribute to the dental follicle, and also the apical part of the papilla, where the roots will ultimately develop. Some cells in the presumptive dental papilla at the cap stage contribute to the follicle at the bell stage, indicating that the dental papilla and dental follicle are still not defined populations at this stage. These lineage-tracing experiments highlight the difficulty of targeting the papilla and presumptive odontoblasts at early stages of tooth development. We show that at the cap stage, cells destined to form the follicle are still competent to form dental papilla specific cell types, such as odontoblasts, and produce dentin, if placed in contact with the inner dental epithelium. Cell fate of the dental mesenchyme at this stage is therefore determined by the epithelium.

Introduction

Mammalian teeth develop from oral epithelium and neural crest derived mesenchyme (Chai et al., 2000) with later immigration of mesoderm derived endothelial cells (Nait Lechguer et al., 2008; Rothová et al., 2011). The epithelium invaginates into the surrounding mesenchyme and forms a dental bud. Later the epithelial cervical loops grow further into the condensed mesenchyme forming a cap and then a bell shaped enamel organ (Fig. 1(A) and (B)). The dental papilla forms in between the cervical loops, in an area that lies in the body of the enamel organ (Fig. 1(C)). The condensed dental mesenchyme surrounding the bud and cap stage epithelium is labelled by the expression of *Syndecan-1* (Vainio et al., 1989, 1991; Fig. 1(A)). The dental papilla is generally assumed to originate passively — from the condensed mesenchyme captured in between the elongating body of the cap or bell shaped enamel organ (as schematized for example in Bite-it.helsinki.fi, Aberg et al. (2004), Tucker and Sharpe (2004) and Fig. 1(B)). The dental papilla later becomes the dental pulp with its associated blood vessels and nerve supply. The remaining condensed dental mesenchyme surrounding the outer enamel organ and dental papilla gives rise to the dental follicle, which later forms the periodontium: cementoblasts, periodontal ligament and alveolar bone (Ten Cate et al., 1971; Yoshikawa and Kollar, 1981; Palmer and Lumsden, 1987; Diep et al., 2009). At the bell stage of tooth development, the dental follicle, or dental sac, is clearly divided into three layers — the inner layer adjacent to the outer surface of enamel organ and dental papilla, the outer layer adjacent to the developing alveolar bone, and a less densely populated intermediate layer separating the other two. The inner layer is probably the most important part of the dental follicle for formation of cementoblasts and periodontal ligament (Ten Cate et al., 1971). The outer layer later contributes to formation of the

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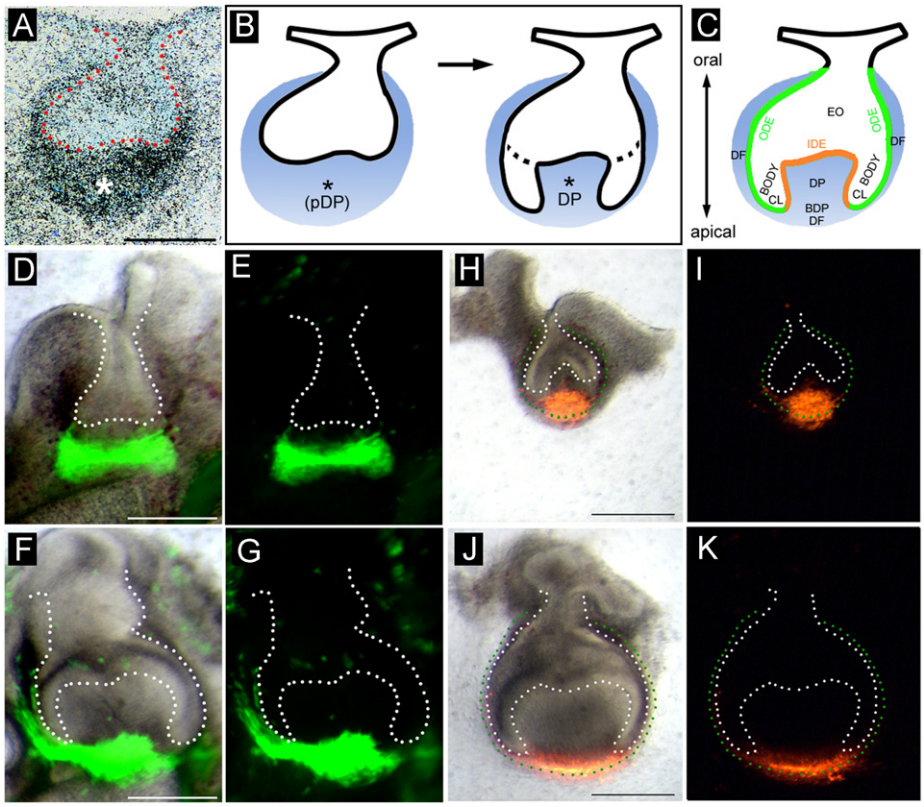

Fig. 1. The condensed dental mesenchyme and origin of the dental papilla in the mouse. (A) Radioactive *in situ* hybridization of *syndecan-1* mRNA in the first lower molar at E14.5. There is a strong *syndecan-1* expression showing the condensed dental mesenchyme in black. The dental epithelium is highlighted by red dotted line. The white asterisk shows a region of the dental mesenchyme that is assumed to become the dental papilla, where some of the GFP grafts and DiI labelling were placed ((D)–(K)). (B) Scheme showing the assumed mechanism of dental papilla origin from the condensed dental mesenchyme (blue). The prospective dental papilla (pDP) represents a part of the mesenchymal condensation, which becomes passively enveloped by the extending dental epithelium. The enfolded passive mesenchymal cell population becomes the dental papilla — DP. (C) Schematic of a molar tooth germ at the late cap stage showing the components of the condensed dental mesenchyme: BDP – apical base of the dental papilla, DF – dental follicle, DP – dental papilla; and of the dental epithelium: CL – cervical loop and the body of the enamel organ, EO – enamel organ, IDE – inner dental epithelium in orange, ODE – outer dental epithelium in green. (D) GFP positive dental mesenchyme was grafted into the presumed DP region (the *syndecan-1* positive condensed dental mesenchyme showed with white asterisk in (A)) of a CD1 molar tooth slice at E14.5, leaving a small unlabelled mesenchymal region next to the IDE. (E) Outline of the epithelium of the same molar tooth germ under dark field. (F) The same molar tooth germ after 3 days of *in vitro* culture. The GFP labelled mesenchyme did not contribute to the main body of the forming dental papilla but ends up at the apical end, at the base of the dental papilla of the bell stage enamel organ. (G) Outline of the epithelium of the same molar tooth germ under dark field. (H) A sliced CD1 mouse mandible containing a molar tooth germ at the cap stage at E14.5. The periphery of the condensed mesenchyme (green dotted line) was labelled with DiI (in red). (I) Outline of the epithelium of the same molar tooth germ under dark field. (J) The same molar tooth germ after 2 days of *in vitro* culture. The DiI labelled cells were located at the base of the dental papilla but did not contribute to the formation of the main body of the papilla. (K) Outline of the epithelium of the same molar tooth germ under dark field. The dental epithelium is highlighted with a white dotted line. ((A); (D)–(K)) Scale is 250 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alveolar bone (Diekwisch, 2002; Diep et al., 2009). When the dental papilla is combined with the enamel organ at the bell stage (embryonic day 16.0) of tooth development, a new inner dental follicle layer forms, originating from the dental papilla cells (Yoshikawa and Kollar, 1981; Palmer and Lumsden, 1987). Tritiated thymidine labelling studies of the third molar at the late bell stage in postnatal mice also indicated a contribution of the papilla to the follicle at a stage when crown morphogenesis is almost complete (Osborn and Price, 1988). However, it remains unknown, whether the dental papilla also contributes to the developing dental follicle during earlier prenatal development.

There have been many extensive studies of the molecular mechanisms that drive tooth development (for review see *e.g.,* Thesleff and Mikkola, 2002; Cobourne and Sharpe, 2010). However, a recent study has pointed out the dynamic nature of dental mesenchyme during tooth morphogenesis, with the condensed mesenchyme adjacent to the epithelial bud contributing to the dental follicle and alveolar bone (Diep et al., 2009). The observed dynamic nature of the dental mesenchyme raises a question, whether the condensed mesenchyme indeed plays only a passive role during papilla origin. Therefore, this present study has focused on the origin of the dental papilla and its developmental relationship to the condensed dental mesenchyme, dental follicle and dental epithelium in the developing mouse lower first molar. The dynamics of the dental mesenchyme was investigated by vital cell labelling and tissue grafting in *in vitro* slice culture.

Material and methods

Mice

CD1 mice and GFP (Green fluorescent protein) mice were used in this study. The females were mated overnight and noon after the detection of the vaginal plug was considered as embryonic day (E) 0.5. Embryos were harvested after the pregnant mice were killed using a schedule 1 method (cervical dislocation) as approved by the Home Office and King's College London.

Slice culture in vitro

E13.5, E14.5 and E15.5 mouse mandibles of CD1 embryos were dissected out and sliced using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Ltd. UK) into frontal slices 250 μm

thick (Diep et al., 2009; Rothová et al., 2011). Slices showing a distinct lower first molar tooth bud or cap were then selected for further processing. Slices were cultured on transparent nucleopore filters (VWR) supported on metal grids on the surface of the medium. Medium consisted of Advanced DMEM F12 (Invitrogen) supplemented with 1% penicillin/streptomycin, 1% Glutamax (Invitrogen). In some experiments (Fig. 1(D)–(G); Fig. 2(E)–(H); Figs. 4 and 6; Suppl. Fig. 1), 10% foetal calf serum was added to the cultures. Matrigel basement membrane matrix (7 µl) (BD Biosciences) was added on top of the slice. Slices were cultured at 37 °C/5% CO_2 up to 6 days, changing the medium every 2 days.

DiI & DiO labelling

DiI and DiO are lipophilic dyes, which intercalate in the cell membrane, marking groups of cells. DiI (Molecular probes cell tracker CM-DiI, C-7000, Invitrogen) and DiO (Molecular probes, V22886, Invitrogen) was dissolved in 100% EtOH. Small amounts of DiI were injected into the slices before culture using a mouth pipette or injector Picospritzer III (Intracel). The slices were then placed on filters and cultured. The position of the DiI/DiO was recorded using a fluorescence Leica dissecting microscope every 24 h.

GFP electroporation

An avian vector pCAß-IRES-mGFP that expresses a myristylated EGFP was electroporated into tissue slices containing tooth germs at E13.5 using tungsten wire electrodes (0.1 mm; Goodfellow, W005138/13), Electro-Square-Porator ECM 830 (Genetronics) and a pulse monitor BTX enhancer 400. A voltage of 25 V and four pulses of 50 ms duration were used. The tissue slices were then cultured *in vitro* as described above.

GFP and CD1 tissue recombination

GFP (Green fluorescent protein) reporter mice were mated, at the same time as CD1 WT mice. GFP and WT mandibles were dissected out at the bud/cap stage and sliced using a tissue chopper (see above). GFP slices were then dispased (Dispase, 2 U/ml, Gibco) to separate the dental epithelium and mesenchyme. The isolated condensed dental mesenchyme from a GFP mouse was then cut into segments of dental papilla (lying directly under the developing inner dental epithelium) and dental follicle (lying directly adjacent to the outer dental epithelium) using fine tungsten needles. For homotopic grafts, a small piece of tissue was then inserted into the corresponding place of the condensed dental mesenchyme of a WT sliced tooth germ, where an equivalent area of condensed mesenchyme had been removed. For heterotopic grafts, dental follicle was grafted into the position of the dental papilla, under the forming inner dental epithelium. After approximately 6–12 h, once the grafted tissue had started to integrate, the culture was photographed under fluorescence to check the position of the grafted tissue. Grafts were then left to develop for several days while the tooth germ developed to the late bell stage.

BrdU injections

CD1 pregnant females were injected intraperitoneally with 20 mg of BrdU per 1 kg weight of the injected animal. The mothers

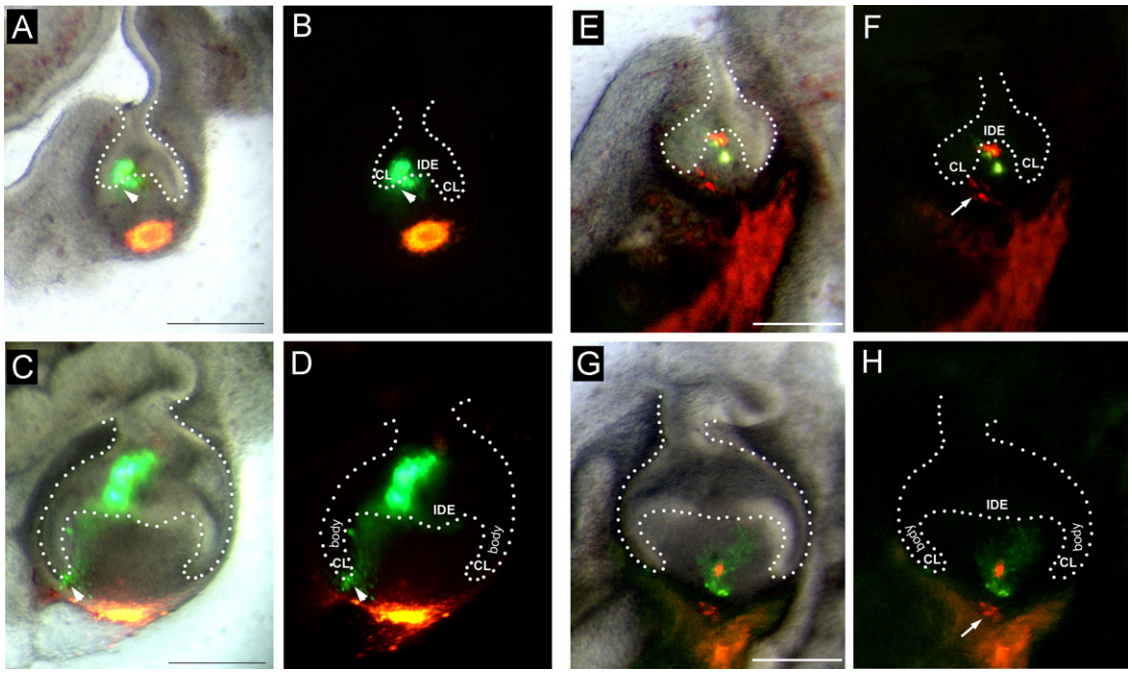

Fig. 2. Cell labelling and fate mapping of the dental mesenchymal cells and epithelium at E14.5. (A) A sliced mouse mandible containing a tooth germ at the cap stage at E14.5. At day 0 a single spot of DiO (green) labels the inner dental epithelium and adjacent mesenchymal cells. A cell population at the periphery of the condensed dental mesenchyme was labelled with DiI (red). (B) Outline of dental epithelium of the same molar tooth germ under dark field. (C) After 2 days of *in vitro* culture, the single labelled region of mesenchymal cells had spread as a band of green DiO labelled cells spanning across the dental papilla. The green labelled dental mesenchyme remained in close contact with the cervical loop (white arrowheads), moving towards the red labelled cells in the forming dental follicle. The DiI labelled cells spread in a layer of the dental follicle adjacent to the base of dental papilla. The labelled epithelial cells spread across the enamel organ. (D) Outline of the same molar tooth under dark field. (E) A sliced mouse mandible containing a tooth germ at cap stage at E14.5. Different positions of the dental mesenchyme were labelled with DiI (red) and DiO (green). (F) Outline of dental epithelium of the same molar tooth germ under dark field. (G) After 2 days of *in vitro* culture, only cells originally located in between the body of the enamel organ contributed to the formation of the dental papilla. Note, that the focal spots of the labels keep their relative positions during tooth development. The white arrows highlight the DiI labelled mesenchymal cell region that do not contribute to the formation of the dental papilla but label the dental follicle. CL – cervical loop, IDE – inner dental epithelium. The dental epithelium is highlighted with a white dotted line. Scale is 250 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were sacrificed 2 h after injection and the E14.5; E15.5 embryos were collected and weighted (Peterka et al., 2002). The embryonic heads were fixed in 4% paraformaldehyde (PFA) over night and embedded in paraffin. The embryonic heads were then sectioned at 10 μm.

BrdU statistical analysis

BrdU index was counted as a proportion of BrdU labelled cells within a total number of cells at various regions. We compared BrdU index between dental papilla and dental follicle regions (as schematized in Fig. 5(A)). The analysis was done on digitalized images (Leica SP5) of serial frontal sections ($n=32$ at E14.5; $n=32$ at E15.5).

Fischer's exact test was used to determine the statistical significance between the total values.

Immuno-fluorescent staining

Double immuno-stainings were performed on 10 μm thick sagittal and frontal frozen sections (Ki67 and PH3 antibodies, see below) and on paraffin sections (BrdU and PCNA antibodies, see below) of mouse embryonic heads. The CD1 mouse embryos at E14.5 and E15.5 were weighted (according to Peterka et al. (2002)), heads were dissected and frozen immediately in Cryomount (HI-00890) or fixed, dehydrated and embedded in paraffin. The frozen sections made using a Microm HM 560 were post-fixed in 4% PFA (10 min), blocked in 10% BSA (30 min) and incubated at 4 °C over night with primary antibody Ki67 (Rabbit polyclonal IgG to Ki67, Abcam, #Ab15570) (diluted 1:1000 in 1% BSA) and primary antibody PH-3 (Mouse monoclonal IgG to phosphohistone-3, Abcam, #Ab14955) (diluted 1:200 in 1% BSA). The secondary anti-rabbit antibody (Alexa Fluor® 488 goat anti-rabbit IgG, Invitrogen, #A-11008) was diluted 1:250 in 1% BSA, the secondary anti-mouse antibody (Rhodamine TRITC sheep anti-mouse IgG, Jackson ImmunoResearch, #515025003,) was diluted 1:150 in 1% BSA and both antibodies incubated at the same time at RT for 1 h. Nuclei were stained with Hoechst (Sigma, #B2261) and the slides mounted in Prolong® Gold anti-fade reagent with DAPI (Invitrogen, #P36935). The images were taken using inverted fluorescence microscope Leica AF6000. The paraffin sections were de-paraffinized, re-hydrated, boiled in 10 mM sodium citrate pH 6 for 5 min 4 ×, blocked with 0.2% gelatine and 10% lamb serum for 2 h and incubated at 4 °C over night with primary antibody PCNA (mouse monoclonal IgG to Proliferating Cell Nuclear Antigen, Sigma, #P8825) (diluted 1:200 in block solution) and BrdU (Rat monoclonal IgG to Bromodeoxyuridine, Genetex, #GTX26326) (diluted 1:100 in block solution). The secondary antibodies (Alexa Fluor® 488 goat anti-mouse IgG, Invitrogen, #A-11001 and Alexa Fluor® 568 goat anti-rat IgG, Invitrogen, #A-11077) were diluted 1:400 in block solution and incubated for 1 h at room temperature. The slides were mounted in Prolong® Gold anti-fade reagent with DAPI (Invitrogen, #P36935) and the images were taken using confocal microscope Leica SP5.

GFP immunohistochemistry

Grafted cultures were fixed in 4% PFA and taken through a methanol series and embedded in wax via isopropanol and tetrahydronapthalene. Anti-GFP antibody (Abcam, #Ab290) was used at a concentration of 1:500 on paraffin sections, followed by a biotinylated anti-rabbit secondary antibody (Dako) at a concentration of 1:200 and Elite vector stain kit (Vector). GFP was visualised using a DAB reaction (Vector). Slides were counterstained in eosin, with some sections additionally stained with Sirrus red to highlight deposited dentin.

In situ hybridization

Embryonic heads at E14.5 were fixed in 4% PFA over night and dehydrated in methanol series, isopropanol and embedded to paraffin through tetrahydronaphtalene. Paraffin sections were cut at 10 μm. The sections were de-waxed in Histoclear (National Diagnostics, #HS-200), re-hydrated in ethanol series, post-fixed in 4% PFA for 20 min and treated with 10 μg/ml Proteinase K (Sigma) at 37 °C for 10 min. The digestion was stopped by 2 mg/ml glycine (Sigma) for 10 min. The slides were then post-fixed in 4% PFA for 5 min, washed in 0.2 M HCl for 15 min, treated with 0.25% acetic anhydride and 0.1 M triethanolamine pH 8 for 10 min, pre-hybridized in HYB solution (50% formamide, 5 × SSC pH 5.5, 1 × Denhardt's, 0.1% Tween 20, 0.1% Chaps, 50 μg/ml tRNA) at 65 °C for 2 h. The *Dlx2* probe was diluted 1:1000 in the HYB solution and the sections were incubated with 200 μl of the probe solution at 65 °C over night. The next day, the sections were washed in Solution 1 3 × 30 min 65 °C (50% formamide, 5 × SSC pH 5.5, 1% SDS) and in Solution 2 3 × 30 min 65 °C (50% formamide, 2 × SSC pH 5.5, 2% SDS). The slides with sections were then blocked in 10% lamb serum and 1% BBR2 (Boehringer, #1096176) in MAB for 2 h and incubated with anti-DIG Alkaline Phosphatase antibody over night at 4 °C over night (Boehringer, #1093274) diluted 1:2000. The next day the sections were washed in MAB 3 × 10 min, 4 × 1 h at room temperature, washed in NTMT (100 mM Tris HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20). The colour reaction was developed in 3.5 μl/ml BCIP and 4.5 μl/ml NBT in NTMT at room temperature. The sections were then fixed in 4% PFA, dehydrated and mounted in DePex.

Radioactive 35S *in situ* hybridization were performed as previously described by Tucker et al. (1998). Mouse Syndecan-1 plasmid was a kind gift from P. Kettunen.

Results

Fate mapping of the dental mesenchymal cells during dental papilla formation

To observe the fate of the dental mesenchyme during mouse odontogenesis, we used grafts of GFP labelled cells from a GFP reporter mouse and lipophilic dyes (DiI and DiO) to label the condensed dental mesenchyme at embryonic day (E) 14.5. At this stage the tooth germ has reached the cap stage with formation of the primary enamel knot signalling centre at the centre of the inner dental epithelium (IDE) (Jernvall et al., 1998). GFP dental mesenchyme was grafted into an equivalent site within the condensed dental mesenchyme of the lower first molar (M1) of a WT mouse tissue tooth slice (see the position indicated by an asterisk within the *syndecan-1* positive area in Fig. 1(A)). When the graft was placed in this region, leaving a small region of the dental mesenchyme under the epithelial tip unlabelled (Fig. 1(D) and (E)), it was expected that the GFP cells would be incorporated into the dental papilla during further development (Fig. 1(B)). However, after 3 days of *in vitro* culture, the GFP labelled mesenchymal cells did not contribute to the main body of the dental papilla but instead spread out in the layers of the dental follicle and in the apical region of the papilla, in between the cervical loops (compare Fig. 1(F) and (G) to Fig. 1(C)).

To rule out problems with integration of the graft, a similar experiment was repeated using the lipophilic dye DiI (Fig. 1(H) and (I)). DiI was injected into the dental mesenchyme

at a distance from the inner dental epithelium (IDE) at the apical border of the condensed mesenchyme at E14.5 (asterisk in Fig. 1(A) and (B); Fig. 1(H) and (I)). After 2 days of *in vitro* culture, when the tooth germ reached the early bell stage, we found labelled cells spread out apically (*i.e.*, further from the oral cavity) at the base of the papilla (Fig. 1(J) and (K)). Similar to the GFP grafting results, these cells did not contribute to the formation of the main body of the dental papilla, which remained free of labelled cells.

To more finely map the condensed dental mesenchyme we injected small groups of cells within the dental mesenchyme with DiI (red) and DiO (green). DiO was used to label the mesenchymal cells in close contact with the IDE. To ensure that we had labelled the mesenchymal cells right up to the boundary with the epithelium, the DiO dot simultaneously labelled the epithelium and the adjacent dental mesenchyme. A DiI dot labelled the condensed dental mesenchyme at the periphery of the tooth germ, far from the dental epithelium and the enamel knot (Fig. 2(A) and (B)). After 2 days of *in vitro* culture the DiO labelled mesenchymal cells formed a light green band of labelled cells along the body of the enamel organ (Fig. 2(C) and (D)). The labelled mesenchymal cells moved together with the growing cervical loop as its epithelial cells moved towards the DiI spot at the base of the dental papilla. The DiI labelled cells also spread out into the peripheral layer of the dental follicle, around the outer dental epithelium (ODE). The labelled epithelial cells spread within the enamel organ between the stalk and IDE (Fig. 2(C) and (D)).

Similar results were observed when the papilla mesenchyme and adjacent IDE were labelled in the middle part of the tooth cap (Suppl. Fig. 1). To target the whole of the dental papilla, GFP labelled tissue was grafted in between the cervical loops, close to the forming inner dental epithelium. After 6 days of *in vitro* culture, the GFP positive cells contributed to almost the whole of the dental papilla (Suppl. Fig. 2).

To precisely follow the fate of the dental mesenchymal cells, we labelled cells at different positions within the dental mesenchyme at E14.5 with DiI and DiO (Fig. 2(E) and (F)). Mesenchymal cells labelled by DiI at of the level of the extending cervical loops (Fig. 2(E) and (F)), formed the peripheral layer of the dental follicle at the late cap stage (after 2 days of *in vitro* culture) but did not take part in formation of the papilla (Fig. 2(G) and (H)). Cells labelled in the mesenchyme within the body of the enamel organ and those adjacent to the IDE and enamel knot at E14.5 were located within the dental papilla, and retained their relative positions as the papilla formed and expanded (Fig. 2(E)–(H)).

To confirm that only specific cells of the condensed dental mesenchyme contribute to the formation of the dental papilla, we labelled two different regions of the dental mesenchyme in the same E14.5 molar tooth germ with DiI. One labelled region (a) comprised dental mesenchymal cells located adjacent to the IDE. The other region (b) comprised mesenchymal cells located slightly further away at the level of the cervical loop. An unlabelled region of mesenchyme was left in the forming dental papilla (Fig. 3(A) and (B)). After 2 days of *in vitro* culture, the labelled cells in close proximity to the IDE (a) formed a band in the enlarging dental papilla with a focal spot at the base of the dental papilla, at the level of the cervical loop (Fig. 3(C) and (D)). The mesenchymal cells labelled at the level of the cervical loop (b) remained close to the cervical loop and did not contribute to the dental papilla. Importantly, the unlabelled region of mesenchyme expanded as the

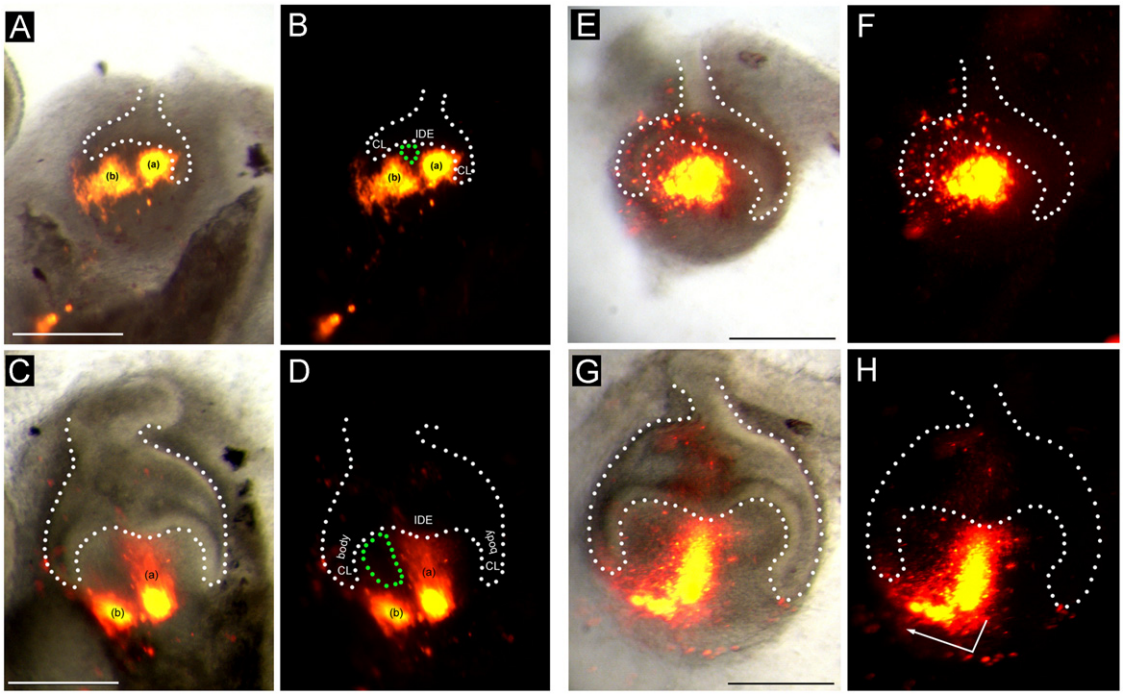

Fig. 3. Cell labelling and fate mapping of different regions of the condensed dental mesenchyme at E14.5 and E15.5. (A) A sliced mouse mandible containing a tooth germ at the cap stage. Two different regions of the dental mesenchyme were labelled with DiI in red. The first DiI labelled region (a) was located in the dental mesenchyme by the IDE. The second DiI labelled region (b) was located in close proximity to the cervical loop (CL) with an unlabelled region left in the dental papilla (shown by green dotted line). (B) Outline of the same molar tooth germ under dark field. (C) The same molar tooth germ after 2 days of *in vitro* culture. The labelled cells originally located in the dental papilla by the IDE (a) formed a band of cells with the focal labelled region staying apically — at the level of the growing cervical loops. The other labelled cells further from the IDE (b) contributed to the dental follicle while the non-labelled region expanded as the dental papilla grew (shown by green dotted line). (D) Outline of the same molar tooth germ under dark field. CL – cervical loop, IDE – inner dental epithelium. (E) A sliced mouse mandible containing a tooth germ at late cap stage, E15.5. Dental papilla cells labelled with DiI in red. (F) Outline of the same molar tooth germ under dark field. (G) After 2 days of *in vitro* culture the labelled cells spread in length as the dental papilla enlarged and elongated. Some cells separated and spread into the dental follicle under the base of the dental papilla (schematized with white arrow). (H) Outline of the same molar tooth germ under dark field. The dental epithelium is highlighted with a white dotted line. Scale is 250 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tooth enlarged during development (Fig. 3(C) and (D)). The dental papilla therefore appears to form from a small population of cells that lie between the forming cervical loops in the body of the enamel organ at the early cap stage.

From our lineage labels at E14.5, the mesenchymal cells near to the IDE and enamel knot, in between the body of enamel organ appeared to rapidly spread out to fill in the whole dental papilla within a few days *in vitro*. We therefore wanted to analyze the fate

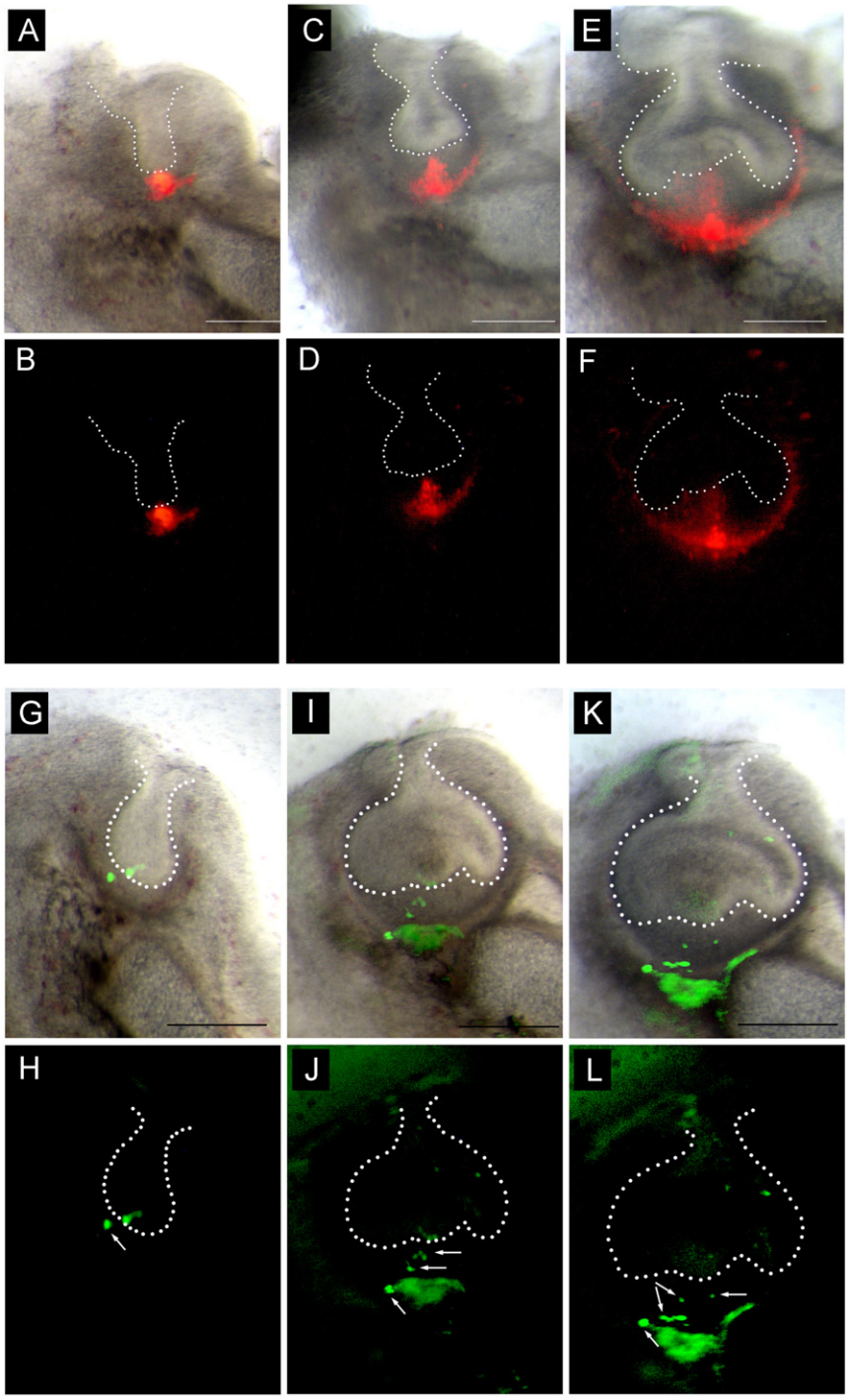

Fig. 4. Cell labelling and fate-mapping of the early condensed dental mesenchyme at the bud stage. (A) A sliced mouse mandible containing a tooth germ at bud stage at E13.5. Mesenchymal cells in close proximity to the tip of the epithelial bud were labelled with DiI in red. (C) The same tooth germ after 1 day of *in vitro* culture. The region of red labelled mesenchymal cells elongated and some cells started to spread into the forming dental follicle. (E) The same tooth germ after 2 days of *in vitro* culture. The labelled mesenchymal cells contributed to the dental papilla but mainly spread further into the dental follicle around the enamel organ. ((B), (D), (F)) Outlines of the corresponding molar tooth germs under dark field ((A) and (B)) day 0, ((C) and (D)) day 1, ((E) and (F)) day 2. (G) A sliced mouse mandible containing a tooth germ at bud stage at E13.5. Dental mesenchymal cells electroporated with a GFP expression construct (white arrow in (H)) are located close to the tip of the dental epithelium but they are not directly adjacent to the epithelium. (I) The same tooth germ after 1 day of *in vitro* culture. The cells expressing GFP distributed into layers of the forming dental follicle (white arrows in (J)). (K) The same tooth germ after 2 days of *in vitro* culture. The GFP cells contribute to the dental follicle (white arrows in (L)). ((H), (J), (L)) Outlines of the corresponding molar tooth germs under dark field at (H) day 0, (J) day 1, (L) day 2. At the site of electroporation a patch of auto-fluorescence develops. The white arrows indicate and distinguish GFP positive cells from auto-fluorescent tissue. The dental epithelium is highlighted with a white dotted line. Scale is 250 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the dental papilla cells slightly later in development at the late cap stage, E15.5. A large group of cells at the centre of the forming dental papilla were labelled with DiI. The labelled area was in close proximity to the IDE in between the body of enamel organ (Fig. 3(E) and (F); compare to Fig. 1(C)). After 2 days of *in vitro* culture the labelled cell population spread as the dental papilla enlarged and elongated in the apical direction (Fig. 3(G) and (H)). In contrast to our labelling at E14.5 (Fig. 3(A)–(D); Suppl. Fig. 1), the E15.5 dental papilla cells did not exhibit the distinct shift towards the base of the dental papilla but remained proportionally inside the dental papilla. Interestingly, some cells at the base of the dental papilla separated and spread into the dental follicle (Fig. 3(G) and (H)), indicating that the papilla cells also contribute to the dental follicle at the late cap stage of tooth development.

The dynamics of dental mesenchymal cells surrounding the tooth germ was even more apparent when the DiI labelling was initiated at the bud stage, E13.5. The dynamic movement of the mesenchymal cells on either side of the tooth bud have previously been shown, with the cells contributing to the dental follicle (Diep et al., 2009). Here we labelled the condensed dental mesenchyme at the tip of the bud in close proximity to the epithelium with the aim of targeting the presumptive dental papilla (Fig. 4(A) and (B)). This region of mesenchyme lies under the prospective primary enamel knot of the first molar (Jernvall et al., 1998). After 1 day of *in vitro* culture the region of labelled mesenchymal cells elongated and some cells spread into the forming dental follicle (Fig. 4(C) and (D)). After 2 days of *in vitro* culture, when the tooth germ had reached the cap stage, the labelled cells took part in the formation of the dental papilla but also in the developing dental follicle surrounding the enamel organ (Fig. 4(E) and (F)). At the bud stage, therefore, the mesenchymal cells under the enamel knot contributed to both the papilla and follicle.

We used GFP electroporation as a more precise cell labelling technique to follow very small populations of cells. When a small region of dental mesenchyme was electroporated slightly further from the tip of the dental epithelial bud at E13.5 (Fig. 4(G) and (H)), we observed this small population of cells contributing to the different layers of the dental follicle (Fig. 4(I)–(L)).

Cell proliferation of the dental mesenchyme

To investigate if there are any specific proliferative domains in the dental mesenchyme which would be responsible for expansion of the mesenchymal cells that form the dental papilla we used four markers of proliferation to precisely map the proliferation pattern. Ki67 antigen is present in cell-cycle active cells and absent from G0 resting cells, similarly PCNA antigen is expressed in all proliferating cells. Phosphohistone-3 (PH-3) is a mitotic marker, which is present in actively dividing cells and a short term BrdU pulse into pregnant females allows replicating cells to be distinguished. Cell proliferation was analyzed along the anterior–posterior length of the developing lower molar on sagittal and serial frontal sections of E14.5 and E15.5 mouse embryonic heads (Fig. 5; Suppl. Fig. 3).

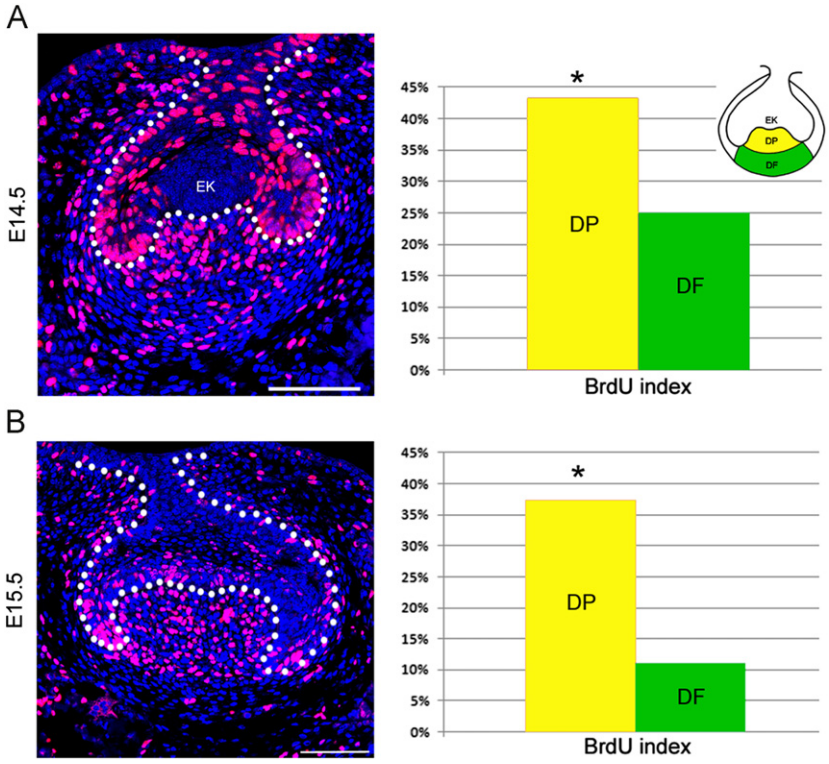

Fig. 5. Study of cell proliferation at E14.5 and E15.5 using short-term BrdU pulses and BrdU antibody labelling. (A) Frontal section of E14.5 molar tooth germ (embryonic body weight 280–310 mg). Immuno-histochemistry of BrdU antibody shown in pink, nuclei stained by DAPI in blue. The dividing mesenchymal cells labelled by a short term BrdU pulse are located mainly in the dental mesenchyme in close proximity to the enamel knot (EK), where the dental papilla develops. The dental epithelium is outlined with white dotted line. Scale is 100 μm. The statistical analysis of BrdU positive cells in dental papilla (schematized in yellow) and dental follicle (schematized in green) at E14.5 shows, that the cell proliferation is statistically higher in the dental papilla region ($p < 0.01$) than in the dental follicle. (B) Frontal section of E15.5 molar tooth germ (embryonic body weight 500–530 mg). Immuno-histochemistry of BrdU antibody shown in pink, nuclei stained by DAPI in blue. There is a clear distinguishable border between the highly proliferating dental papilla compartment (in between the body of enamel organ) and the low proliferating dental follicle cells exhibiting minimum BrdU labelled cells. The dental epithelium is outlined with white dotted line. Scale is 100 μm. The statistical analysis of BrdU positive cells in dental papilla and dental follicle at E15.5 shows, that the cell proliferation is statistically higher in the dental papilla (in yellow) region ($p < 0.01$) than in the dental follicle (in green). DF – dental follicle, DP – dental papilla, EK – enamel knot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

By this extensive proliferation study we revealed that short term BrdU labelling is the best marker for distinguishing the highly proliferating dental papilla cells at E14.5 and E15.5 (Fig. 5). In contrast to the other proliferation markers (Suppl. Fig. 3), the majority of the BrdU labelling was present specifically in the mesenchymal cells adjacent to the enamel knot, in between the body of the enamel organ at E14.5 and E15.5 (Fig. 5(A) and (B)). In contrast, the rest of the condensed dental mesenchyme (surrounding the enamel organ and enclosed papilla) showed a significantly lower level ($p < 0.01$) of cell proliferation using BrdU. As a result, a clear border was apparent between the dental papilla and the rest of the dental mesenchyme (Fig. 5(A) and (B)).

Fate verses determination

Our fate mapping experiments have defined the mesenchymal cells that take part in the formation of the dental papilla and follicle. Whether the dental mesenchyme is determined to form these structures at the cap stage, however, was unclear. To investigate this, GFP labelled follicle cells were grafted into the presumptive dental papilla region at the cap stage to assess whether these cells could be recruited to form the dental papilla at the bell stage. The results of these grafts were compared to similar sized grafts, where the presumptive dental papilla had been grafted into the papilla, and dental follicle into the follicle. When the presumptive dental papilla was grafted into the presumptive dental papilla, the GFP cells integrated, proliferated and filled the majority of the forming dental papilla at the bell stage (Fig. 6(D)–(F) and Suppl. Fig. 2). When the dental follicle was grafted into the dental follicle on the side of the tooth germ, the GFP cells lined the side of the tooth, running under the forming papilla (Fig. 6(A)–(C)). When the dental follicle was grafted into the presumptive dental papilla, the GFP cells proliferated and filled the grafted area of the papilla at the bell stage (Fig. 6(G)–(I)). In section the GFP follicle cells could be seen to fill the papilla, integrating with the host papilla tissue, in a similar manner to the grafted presumptive papilla cells (compare Fig. 6(F)and (I)). The grafted follicle cells differentiated in the host papilla into elongated odontoblasts at the epithelial–mesenchymal border and produced dentin matrix, as stained by Sirrus red (Fig. 6(J)). Therefore the follicle cells formed functional odontoblasts, a cell type only associated with the dental papilla. The dental mesenchyme, although localized to become dental papilla and follicle, was therefore not determined to form these tissues at the cap stage and could be influenced by signals from the local environment after grafting.

Direction of tooth germ growth

Hypothetically, tooth germs can grow in two directions: — in an oral direction, *i.e.,* towards the oral cavity (Fig. 7(A)), or in an apical direction, *i.e.,* growing deep into the surrounding tissues (Fig. 7(B)). Although it is generally assumed that tooth germs grow into the jaw (Fig. 7(B)), measurements of the position of the developing human tooth germs in the maxilla and their surrounding tissue indicated that tooth germs might in fact grow towards the oral cavity with the deepest tip of the dental epithelium staying at a fixed position (Orban 1928) (Fig. 7(A)). As the slice culture *in vitro*

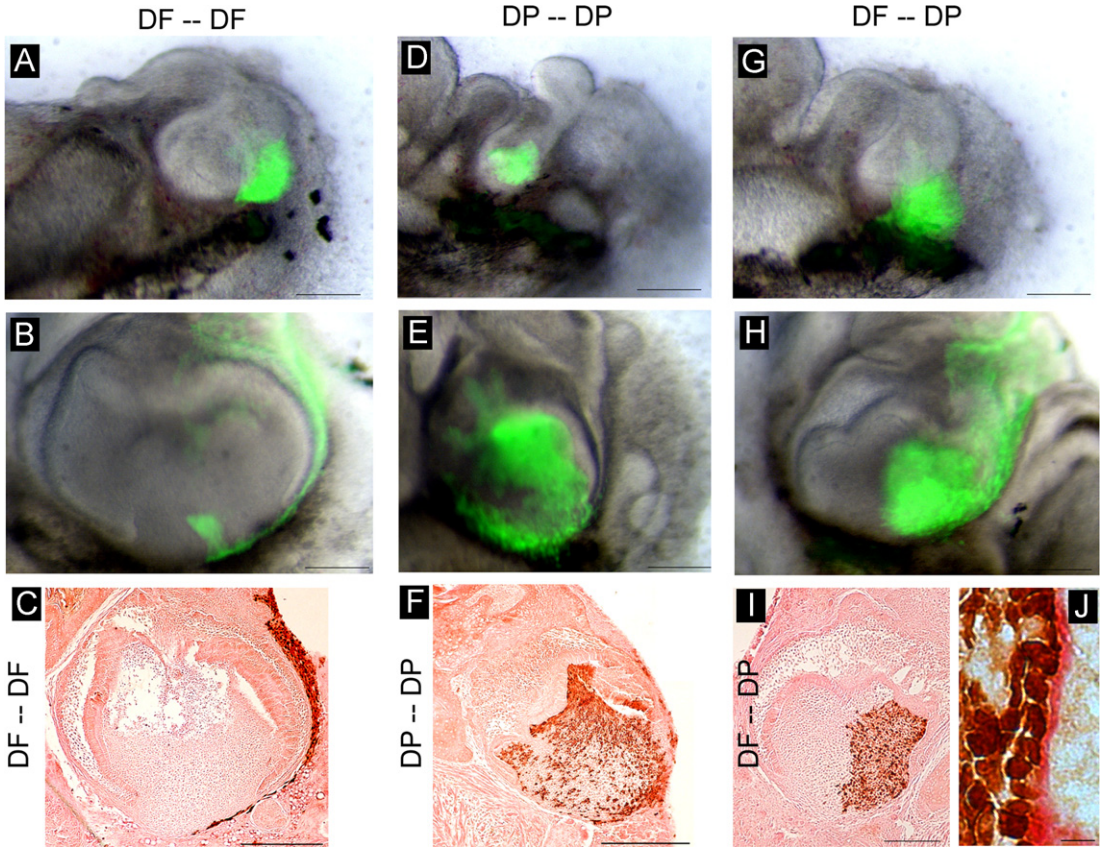

Fig. 6. Plasticity of the dental mesenchyme at the cap stage. GFP grafts into CD1 developing tooth germs at the cap stage. ((A), (D) and (G)) GFP tissue 1 day after grafting. The donor tissue has integrated into the host slice. ((B), (E) and (H)) Grafts after 6 days of culture. ((C), (F) and (I)) Sections through grafts after 7 days in culture stained with an anti-GFP antibody (brown). ((A)–(C)) Dental follicle cells grafted into the dental follicle. ((D)–(F)) Presumptive dental papilla grafted into the dental papilla. ((G)–(I)) Dental follicle cells grafted into the presumptive dental papilla. (J) In section the GFP follicle cells can be seen to form elongated odontoblasts at the epithelial-mesenchymal border, and to secrete a dentin matrix (red stain). ((A)–(I)) Scale is 200 μm. (J) Scale is 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

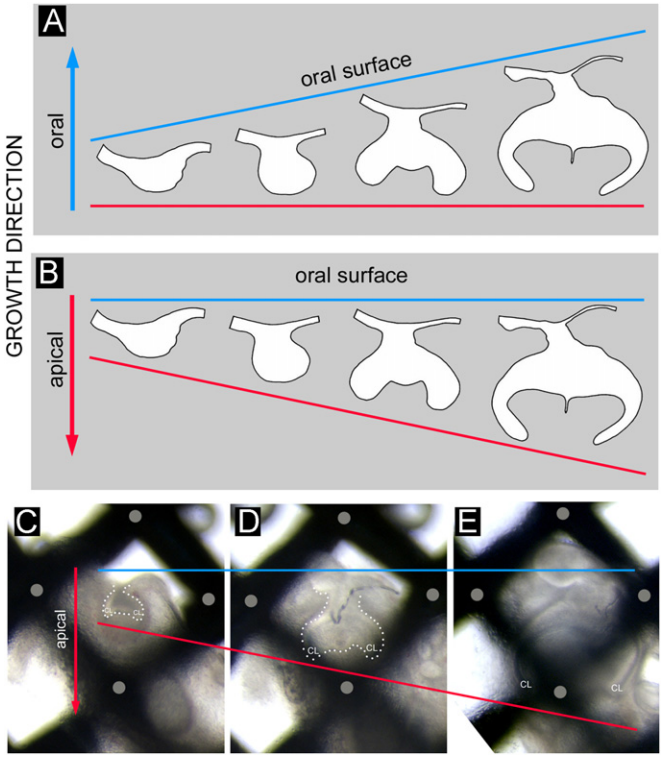

Fig. 7. Directional growth of tooth germs. ((A) and (B)) A scheme of two possible directions of tooth germ growth. Adjusted from Orban (1928). (A) The growth of tooth germ of human maxilla. During odontogenesis, the deepest part of the dental epithelium stays at the same level (red line) while the tooth germ elongates and grows towards the oral cavity surface (blue line). (B) Generally assumed direction of tooth germ growth — the deepest part of the dental epithelium in-grows into the surrounding tissue (red line), while the oral cavity surface stays at the same level (blue line). ((C)–(E)) *In vitro* culture of the mouse lower first molar. The grey points show the stable positions of the grid, which was used as a reference scale. (C) A sliced mouse mandible containing a molar tooth germ at the cap stage (E14.5) was placed on a metal grid. (D) The same molar tooth germ after 1 day of *in vitro* culture. The epithelium has started to in-grow into the surrounding tissues towards the lower grey spot. (E) The same molar tooth germ after 4 days of *in vitro* culture. The epithelial cervical loop (CL) have grown further into the surrounding tissues (red line) to the lower grey spot on the grid, while the dental stalk stayed at a similar position (blue line) during the culture period. CL — cervical loop. The dental epithelium is highlighted with a white dotted line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

method provides a natural environment for the tooth to grow with the tissues surrounding the tooth germ, we could investigate the growth direction of the lower mouse molar *in vitro*. We cultured E14.5 cap stage tooth germs on a metal grid, which provided fixed reference points as a scale to follow the direction of growth of the tooth germs. After 4 days of *in vitro* culture we observed the cervical loops extending deep into the surrounding tissues with the oral epithelium staying at a similar position during this growth (Fig. 7(C)–(E)). This suggests that the mouse lower molar is not growing towards the oral cavity (Fig. 7(A)) but in-growing into the surrounding tissues, in the apical direction (Fig. 7(B)).

Discussion

In this present study we have re-assessed the origin of the dental papilla and its relationship to the growth of dental epithelium and the body of the enamel organ with the cervical loops at its margin. It has been generally assumed that the dental papilla forms passively from the majority of the early condensed dental mesenchyme enclosed into the cavity of the cap or bell staged enamel organ (Bite-it.helsinki.fi; Aberg et al., 2004; Tucker and Sharpe, 2004; Takatalo et al., 2009; Fig. 1(B), Fig. 8(E)). In contrast, we show that the mesenchymal cells that give rise to the future dental papilla represent a restricted cell population, which at E14.5 can be distinguished from the rest of the condensed mesenchyme by its distinct cellular organization (higher cell density) (Fig. 8(A) and (B)) and high level of cell proliferation (Fig. 8(C)). Thus, at this early stage, the condensed dental mesenchyme is already divided into distinct compartments with distinct fates. The boundary of the dental papilla at the cap stage appears to be between the tips of the cervical loops, with cells within the body of the enamel organ forming the majority of the dental papilla, while those outside of this region form the follicle. The cells that will form the dental papilla are therefore very closely associated with the inner dental epithelium and primary enamel knot.

The mesenchyme, however, is not yet determined to form specific cell types, and our heterotopic grafting experiments show that follicle cells explanted at the cap stage can form papilla specific cell types if grafted into the host papilla and given the correct signals. These signals are likely to be driven by the inner dental epithelium, in particular the primary enamel knot that forms at the late bud stage (Jernvall et al., 1998; Prochazka et al., 2010). The dental mesenchyme at the cap stage therefore retains its plasticity, which is shaped by the local environment.

It has recently been demonstrated that the dental mesenchymal cells around the tooth bud move apically as the tooth develops and contributes to the alveolar bone and dental follicle development (Diep et al., 2009). Here we show that the fate of the dental mesenchyme is even more complex. Our present data document that there is only a small population of dental mesenchymal cells at E13.5 and E14.5 that give rise to the dental papilla. Our current results, and previous fate map studies from the lab, show that the majority of the condensed dental mesenchymal cells at E13.5 and E14.5, together with the dental papilla cells themselves, contribute to the formation of the dental follicle. Importantly, the condensed dental mesenchyme located under the tip of the bud shaped dental epithelium at E13.5 is capable of populating the dental papilla, and dental follicle on either side of the enamel organ. We show that the dental papilla cells contribute to the dental follicle from the cap to bell transition stage of the first molar development. These findings supplement the previous grafting and auto-radioactive studies, which suggested that dental papilla cells could contribute to development of the dental follicle during later stages of odontogenesis (Yoshikawa and Kollar, 1981; Palmer and Lumsden, 1987; Osborn and Price, 1988). Our labelling studies also suggest that the dental papilla mesenchyme from E15.5 (late cap stage) is significantly more suitable for studies aiming to target the dental papilla and its derivatives, as the mesenchymal cell population at E15.5 is much larger, more clearly defined and stable compared to earlier stages. Targeting of the condensed dental mesenchyme at E14.5 or earlier could quite easily miss the cell population of the developing dental papilla completely, and label substantial parts of the dental follicle. This helps to explain the technical problems of separating the dental papilla from the follicle at E14.5 as reported elsewhere (Kim et al., 2007).

It could be argued that the results of the present DiI/DiO labelling are caused by directional growth of the dental epithelium towards the oral cavity, while the dental mesenchyme stays at the same position, expands and grows together with the epithelium towards the oral cavity, as was reported during development of human maxillary teeth (Orban, 1928; Fig. 7(A)). This hypothesis could have explained why we see bands of labelled cells in the forming dental papilla and why the main labelled focal spot is located by the deepest tip of the dental epithelium (by the cervical loops) at the end of most of our fate-mapping experiments. However, when we investigated the

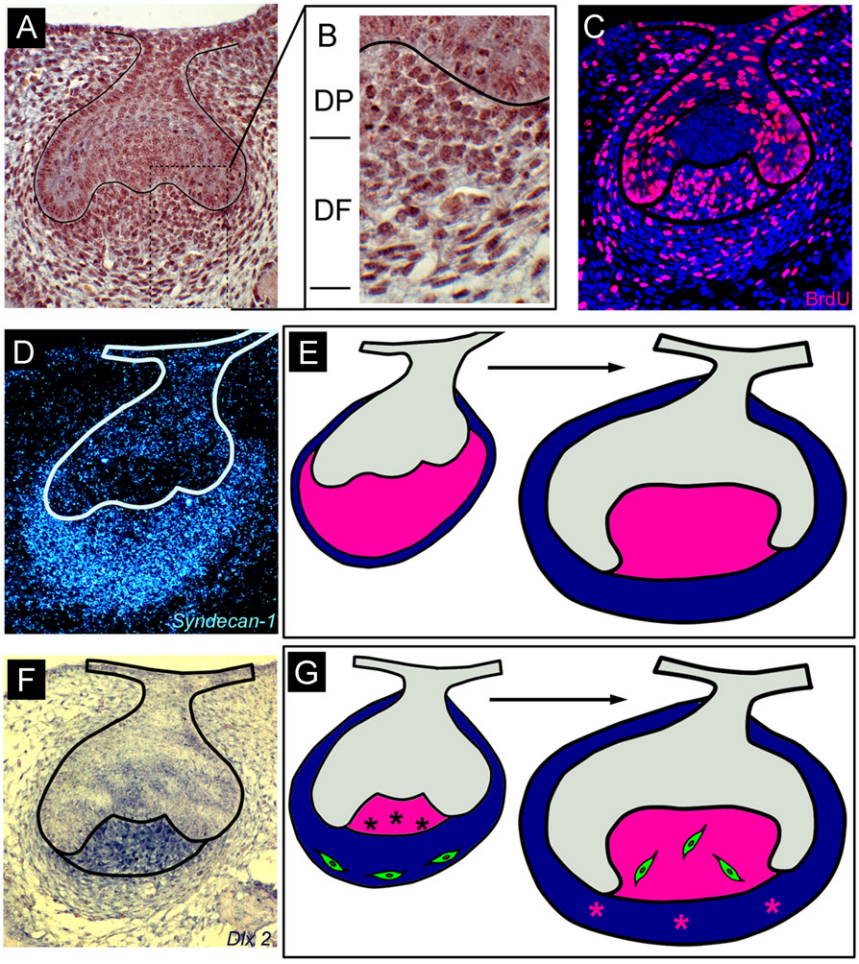

Fig. 8. Revised concept of the dental papilla origin. (A) Histological section of E14.5 molar tooth germ at the cap stage. (B) Higher magnification for better resolution of the dental mesenchymal cell compartments. Already at the early cap stage, there are two distinguishable layers (black lines) of dental mesenchyme. Note, that the cells of the presumptive dental papilla (DP) are more compact than in the dental follicle compartment (DF). (C) Immuno-histochemistry of BrdU, showing high cell proliferation (pink) in the developing dental papilla — adjacent to the non-proliferating region of the enamel knot. (D) The first lower molar at E14.5. Radioactive *in situ* hybridization of *syndecan-1*, showing the condensed dental mesenchyme (light blue grains). Dark field. (E) Generally accepted concept of dental papilla origin, where the majority of the dental mesenchyme represents the prospective dental papilla (in pink) (characterized by *syndecan-1* expression at E14.5 cap stage) while the prospective follicle (in blue) constitutes only a small population of cells lining the enamel organ and presumptive dental papilla (Bite-it.helsinki.fi; Aberg et al., 2004; Tucker and Sharpe, 2004; Takatalo et al., 2008, 2009). (F) The first lower molar at E14.5 and *in situ* hybridization of *Dlx2*, showing a restricted region of the condensed dental mesenchyme (blue), corresponding to the BrdU positive region (pink in (C)) and the compact cell layer (DP) (in (A) and (B)). (G) Our revised concept of dental papilla origin. Lineage labelling shows that the dental papilla develops from only a limited mesenchymal cell population (in pink), located adjacent to the enamel knot and IDE in between the body of the enamel organ. The rest of the dental mesenchyme forms the dental follicle (blue). At the bell stage, the mesenchymal papilla cells (pink) fill up the space in between the body of the enamel organ. Follicle cells spread around the enamel organ, with some dental papilla cells at the cap stage (black *) moving out from the papilla into the follicle at the bell stage (pink *). At the early cap stage, mesoderm derived endothelial cells (in green) are already located at the periphery of the dental follicle and later immigrate into the papilla region (Rothová et al., 2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

direction of growth of the cultured first molar on a metal grid, we found significant in-growth of the epithelial cervical loops in the apical direction. Thus the present data strongly suggest that the bands of the labelled cells result from dispersion of the dye as the labelled highly proliferating dental papilla cells divide while the lower first molar in-grows into the surrounding tissues, with the epithelial cervical loops growing together with the restricted dental papilla mesenchyme. This mutual growth of the dental epithelium and mesenchyme is supported by an earlier study, where the growth of the mesenchymal component (papilla mesenchyme plus free dental follicle mesenchyme) was compared to that of the epithelial component (enamel organ) (Peterková, 1974). In this study, the volume ratio of the dental epithelium and mesenchyme components remains approximately 1:1 during development from the cap to early bell stage (Peterková, 1974). Therefore, this indicates that the dental mesenchyme is not a passive structure enfolded by the faster growing dental epithelium.

At E14.5 and E15.5 we were able to detect specific domains of high cell proliferation within the dental mesenchyme which allowed the different regions of the dental mesenchyme to be distinguished. The restricted region of the developing dental papilla is a highly proliferative area of more densely arranged cells, while the surrounding dental follicle mesenchyme exhibits low number of proliferating, less packed cells. Low epithelial cell proliferation was present in the epithelial region of the primary enamel knot signalling centre as described previously (Jernvall et al., 1994). The enamel knot expresses many signalling molecules (Shh, Fgf4, Bmp4, Wnts) involved in epithelium-mesenchyme reciprocal interactions that drive tooth development (for review see Jernvall and Thesleff, 2000). We propose a model where the mesenchymal cells in close proximity to this epithelial signalling centre obtain the appropriate signals sent from the enamel knot and drive formation of the dental papilla, while cells further away, which do not reach the same molecular information, contribute to the dental follicle.

During the bud and cap stages the condensed dental mesenchyme is morphologically quite a homogenous cell population and gene expression in this region often appears quite uniform, as shown, for example, by *syndecan-1* and others (*e.g.,* Aberg et al., 1997; Aberg et al., 2004, Fig. 8(D)). However, the expression of some genes suggests specific compartmentalisation of the dental mesenchyme at the cap stage. For example expression of *Dlx* genes shows some specific compartmentalization (Zhao et al., 2000) and together with *Fgf3* (Klein et al., 2006) are expressed in a limited proportion of the dental mesenchyme. *Dlx2* (Fig. 8(F)) and *Spry4* (Klein et al., 2006) appear specifically present in the presumptive dental papilla mesenchyme (based on the morphological data — Fig. 8(A) and (B)) at the cap stage. Therefore, *Dlx2* and *Spry4* represent good markers for the forming dental papilla cell population. Our fate map contradicts some claims for specific dental mesenchyme markers. For example, the Golgi protein *GoPro49* has been suggested as a specific marker of the dental follicle from the early bud stage of tooth development (Takatalo et al., 2008, 2009). Although this gene appears an excellent marker of the follicle from the bell stage onwards, our fate mapping data shows that at the cap stage this gene only marks a subset of the dental follicle.

Based on the present data we propose a revised concept of dental papilla origin (Fig. 8(G)) to correct the misleading interpretations from the past (Bite-it.helsinki.fi; Aberg et al., 2004; Tucker and Sharpe, 2004; Takatalo et al., 2009; schematized in Fig. 8(E)). The dental mesenchymal cells adjacent to the inner dental epithelium in between the body of enamel organ at the early cap stage strongly proliferate and fill up the enlarging dental papilla, stimulated by signals from the enamel knot and inner dental epithelium (IDE) (Fig. 8(G)). At the same time the condensed mesenchymal cells at a distance from the IDE proliferate at a lower rate and move around the enamel organ enclosing the papilla and forming the dental follicle but do not take part in the formation of the papilla. As the tooth cap develops, some dental papilla cells move out of the papilla into the follicle (Fig. 8(G)). At the same time as the papilla cells are moving out, recent studies have shown that mesoderm derived endothelial cells immigrate into the developing dental papilla as an influx of non-neural crest cells giving rise to blood vessels (Rothová et al., 2011) (Fig. 8(G)). Dental mesenchyme at early stages of development therefore represents a very dynamic population of cells, with cells moving in and out of the papilla, something perhaps not appreciated from molecular and histological studies. Importantly, the early compartmentalization of the dental mesenchyme can help in the interpretation of molecular data to allow a clearer understanding of early tooth morphogenesis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.03.018.

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