

Contribution of the Tooth Bud Mesenchyme to Alveolar Bone

LISA DIEP¹, EVA MATALOVA^{2,3}, THIMIOS A. MITSIADIS⁴,
AND ABIGAIL S. TUCKER^{1*}

¹*Department of Craniofacial Development and Orthodontics,
King's College London, London, United Kingdom*

²*Laboratory of Animal Embryology, Institute of animal physiology and Genetics,
AS CR, v.v.i., Brno, Czech Republic*

³*Department of Physiology and Pathophysiology, University of Veterinary and
Pharmaceutical Sciences, Brno, Czech Republic*

⁴*Department of Orofacial Development and Structure, Institute of Oral Biology,
University of Zurich, Zurich, Switzerland*

ABSTRACT This study highlights the dynamic nature of the mesenchymal cells during tooth development from the bud to the bell stage. Condensing mesenchymal cells, labelled on either side of the developing tooth bud, move toward the presumptive roots forming an arc of cells under the dental papilla. These labelled cells take part in formation of the dental follicle, which contributes to both the tooth and its surrounding periodontium, including the supporting alveolar bone. This study, thus, physically links development of the tooth with the tissue into which it develops. The results obtained clearly indicate that the tooth organ is an entity comprising dental and periodontal tissue. *J. Exp. Zool. (Mol. Dev. Evol.) 312B, 2009.* © 2009 Wiley-Liss, Inc.

How to cite this article: Diep L, Matalova E, Mitsiadis TA, Tucker AS. 2009. Contribution of the tooth bud mesenchyme to alveolar bone. *J. Exp. Zool. (Mol. Dev. Evol.) 312B*: [page range].

Odontogenesis involves the generation of both dental and periodontal structures. The periodontium comprises the alveolar bone, the periodontal ligament, and cementum. The size and shape of the teeth determine the morphology of the alveolar bone, which continues to develop and undergo remodelling as the tooth progresses and eventually erupts (Cho and Garant, 2000).

Development of the supporting structures of the tooth is an essential part of odontogenesis and closely linked with that of the tooth itself. If a tooth germ is dissected out and cultured subcutaneously, or within the eye or kidney capsule of an adult host, the tooth develops with its supporting structures intact, indicating that the tooth germ contains all the cells to form the periodontium (Ten Cate et al., '71; Ten Cate and Mills, '72; Yoshikawa and Kollar, '81; Palmer and Lumsden, '87; Kratochwil et al., 1996).

The alveolar bone sockets, into which the tooth germs anchor, are evident histologically once the tooth germs have reached the bell stage (Radlanski et al., '98). At this stage of development the mesenchyme of the tooth germ can be divided into the dental papilla, delimited by the inner enamel epithelium, and the dental follicle, which surrounds the outer enamel epithelium (OEE) and lies under the papilla. The follicle is subdivided into three layers: an inner investing layer that is continuous with the lower part of the dental papilla and in close contact with the OEE, an outer layer,

Grant sponsor: Czech Republic; Grant numbers: GA CR 524/08/J032 and GA AV B500450802.

*Correspondence to: Abigail S. Tucker, Department of Craniofacial Development and Orthodontics, King's College London, Floor 27 Guy's Tower, London Bridge, SE1 9RT London, UK.
E-mail: Abigail.tucker@kcl.ac.uk

Received 14 November 2008; Accepted 19 November 2008
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21269

adjacent to the developing alveolar bone, known as the perifollicular layer, and a sparsely populated intermediate layer in between (Palmer and Lumsden, '87; Cho and Garant, 2000). These layers are schematically shown in Fig. 1A. At more advanced developmental stages (i.e. postnatal day 6 (P6)), DiI labelled dental follicle cells form alveolar bone, indicating the contribution made by the follicle layer in forming the alveolar bone (Diekwisch, 2002).

The osteogenic potential of dental mesenchymal cells from less advanced developmental stages (bud and cap) has been shown by tissue culture experiments. At the bud stage of tooth development, neural crest-derived mesenchymal cells start to condense around the invaginating dental epithelium (Chai et al., 2000). If the condensing dental mesenchyme is isolated at the bud stage (E13.5) and cultured in a bone-supporting medium, it can form bone (Yamazaki et al., 2007). In a similar manner, dental follicle cells from a cap stage tooth (E14.5) formed bone when cultured under a kidney capsule for 2 weeks (Kim et al., 2007). This is perhaps unsurprising, given the expression of many genes linked to bone development in the dental

mesenchyme at early stages of tooth development. These genes include *Runx2* (*Cbfa1*), *Dlx5*, *Msx1*, and *Bmp4*, which are all co-expressed later in the developing alveolar bone around the developing tooth at the bell stage (Zhang et al., 2006). In the *Runx2* knockout no bone forms and tooth development is arrested at the cap stage (Ducy et al., '97; D'Souza et al., '99). Whether the mesenchymal cells, which condense around the developing epithelial tooth germ at the bud stage, go on to form alveolar bone at the bell stage of tooth development has so far not been analyzed owing to problems of access to the developing tooth bud. In this study we follow the movements of dental mesenchymal cells from the bud to the late bell stage using a fluorescent dye (DiI) in a tooth slice culture system (Matalova et al., 2005). From such labelling experiments the movements of mesenchymal cells can be visualized, along with their resultant fate.

MATERIALS AND METHODS

Slice preparation

E13.5 mouse mandibles were dissected out, placed upon a chopping plate and orientated to

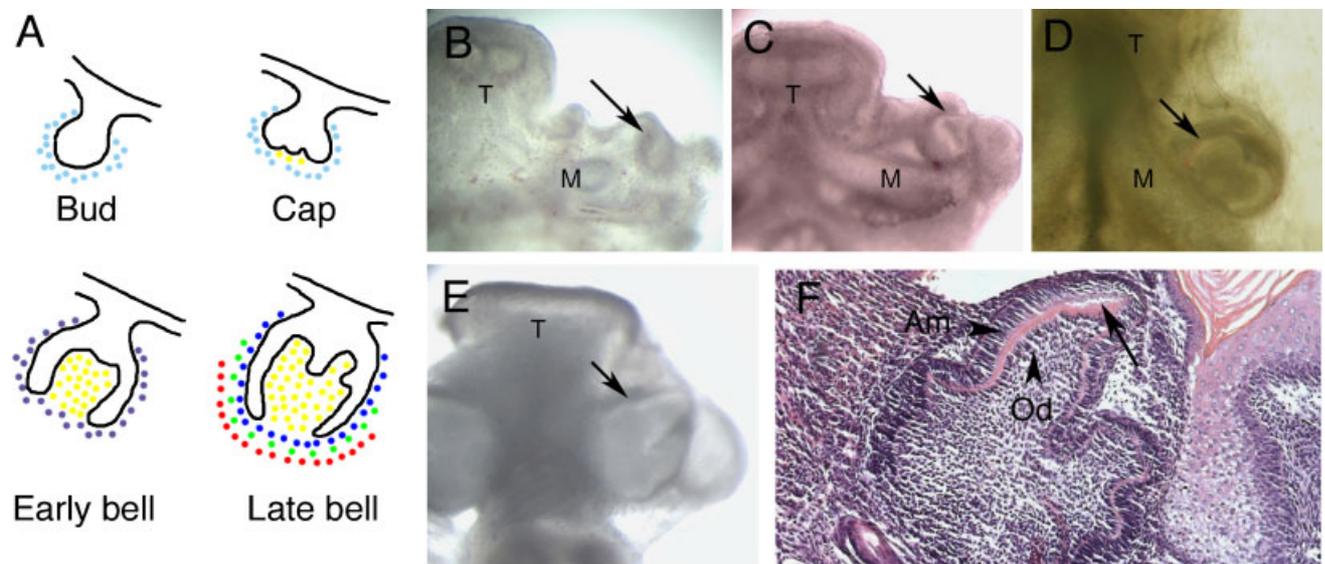


Fig. 1. Molar tooth development in slice culture. (A) Schematic showing the stages of tooth development from bud to late bell. Bud and Cap: light blue spots represent the condensing mesenchyme. Early Bell: purple spots represent the dental follicle. Late Bell: the follicle can be divided into the investing layer (dark blue), the intermediate layer (green), and the outer layer (red). Yellow spots (Cap–Bell) represent the developing dental follicle. (B–F) Frontal slice showing molar tooth germ developing in culture. (B) E13.5 molar tooth germ slice before culturing. The tooth germ is at the bud stage. (C) The same slice after 1 day of culture, showing progression to the early cap stage. (D) After 4 days of culture the tooth progresses to the early bell stage. (E) After 8 days of culture the tooth progresses to the late bell stage. (F) Section through cultured explant in (E) showing complex bell stage invaginations of the epithelium. The odontoblasts (Od) and ameloblasts (Am) have formed and dentin has been deposited (pink stain). Owing to decalcification of the sample for sectioning the presence of enamel can only be inferred from the gap in the tissue between the ameloblasts and dentin (arrowed). (B–E) Tooth germs are highlighted by arrow. T, tongue; M, Meckel's cartilage.

give frontal sections. Using a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Ltd., Guilford, UK), the mandibles were cut into 250 μm thick slices. Slices showing a clear molar tooth bud were then selected and cultured. All animals were killed using a schedule 1 method as approved by the Home Office and King's College London.

DiI labelling

Slices were labelled with 1, 1', di-octadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular probes cell tracker CM-DiI, C-7000) dissolved in EtOH. DiI is a highly lipophilic dye that readily intercalates into the cell membrane. Small amounts of DiI were injected using a mouth pipette into the area of condensing mesenchyme, visible as a dense population of cells around the molar bud. The initial and subsequent positions of the DiI labelled cells were monitored and visualized throughout the culture period under UV light using a Leica dissecting microscope (Leica Microsystems Ltd., Milton Keynes, UK).

Slice culture

Labelled slices were placed upon Millipore filters, which had been coated with Matrigel basement membrane matrix (BD Biosciences, Oxford, UK). Another coat of Matrigel was added on top of the slice so that the slice was completely encapsulated within the Matrigel. This served to structurally support the slice during its development. The filters were supported above the culture medium by metal wire mesh grids within organ culture dishes. The culture medium was composed of Dulbecco's Minimum Essential Medium supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 10% fetal calf serum. Slices were cultured for 6 or 8 days in a 37°C/5% CO₂ air-jacketed incubator.

Fixation and histology

The cultures were fixed in 4% paraformaldehyde for 30 min. Cultures of over a week old were decalcified before embedding in EDTA. Samples were embedded in wax and sectioned at 8 μm . Before staining, sections on slides were photographed under fluorescence to visualize the location of the stable DiI label. The sections were then stained with haematoxylin and eosin or sirrus red and alcian blue and re-photographed.

In situ hybridization

Radioactive S35 in situ hybridization was performed on slices according to Wilkinson ('95). *Syndecan-1* plasmid was a gift from P. Kettunen. *Runx2* plasmid was a gift from Rena D'Souza.

RESULTS

Normal development of tooth germs in slice culture

Slices were taken from the lower jaws of E13.5 day old embryonic mice, by which stage the tooth germ has reached the bud stage. To show the validity of the slice culture method for early tooth development, slices with the bud stage molar anlagen were cultured for 8 days (Fig. 1B–F). During the period of culture the tooth germs developed into clearly recognizable cap and then bell stage tooth germs ($N = 21/21$). The normal process of tooth development was therefore followed in these molar slices (compare slices Fig. 1B–E to schematic development shown in Fig. 1A). Histological sections showed regulated rows of odontoblasts (from the mesenchyme), pre-ameloblasts (from the epithelium), and formation of predentin and dentin (Fig. 1F).

Labelling of the condensing mesenchyme at E13.5

For the purpose of our experiments it was important to show that the condensing dental mesenchyme could be visualized for accurate labelling. In the slices cut at E13.5, a darker patch of cells, surrounding the epithelial tooth bud, was visible (Fig. 2A). When the slices were sectioned at this point, the ring of denser cells overlapped with the condensing dental cells as viewed by histology, and also by the expression of *syndecan-1*, a cell surface heparin-sulphate proteoglycan that marks condensing dental mesenchyme (Vainio et al., '91) (Fig. 2B,C). The expression of *syndecan-1* overlapped with the expression of *Runx2* in the condensing dental mesenchyme, indicating the osteogenic potential of these cells (D'Souza et al., '99). *Runx2* was also expressed strongly in the developing mandibular (dentary) bone. An area of cells between the tooth and developing bone remained free of expression, indicating the independence of these two regions of expression (Fig. 2D). In the cultured slices this region of condensing mesenchyme on either side of the epithelial bud was targeted by injecting small

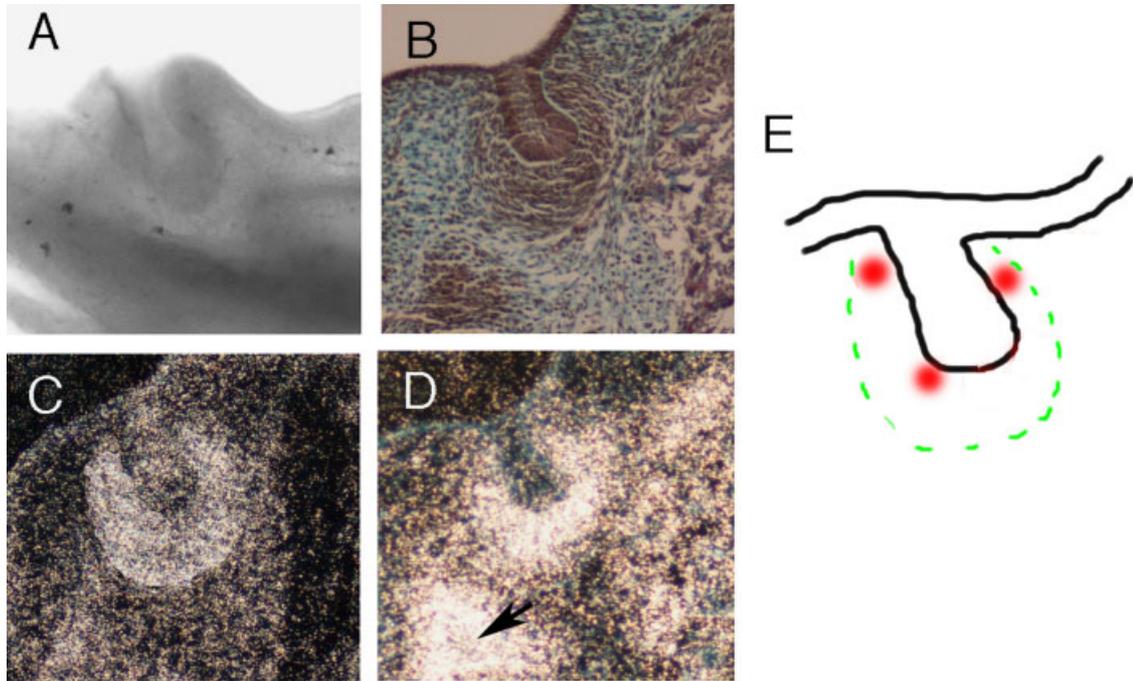


Fig. 2. Targeting of the condensing mesenchyme at the bud stage. (A) A halo of condensing mesenchyme is visible as a darker ring around the E13.5 epithelial bud. (B) Histology at E13.5 on a section through a similar slice. (C) *Syndecan-1* expression around the epithelial bud. (D) *Runx2* expression around the epithelial bud and in the developing dentary bone (arrow). (E) Schematic of bud representing the position of DiI label. Dotted lines represent the boundary of the condensing mesenchyme.

amounts of DiI with a mouth-controlled pipette (position of label schematically shown in Fig. 2E). In order to ensure labelling of the dental mesenchyme the DiI spots were placed as near to the epithelium as possible. The DiI labelled cells were followed over the course of 6–8 days.

The mesenchymal cells labelled close to the epithelium on the lingual and buccal sides at the top and middle of the tooth germ, remained in close contact with the epithelium, following the contour of the developing cervical loops as they extended ($N = 20$) (Fig. 3A–F). In general, cells moved in an apical direction toward the ends of the cervical loops. Cells originally labelled near to the oral surface ended up near the presumptive roots and periodontium of the tooth after 6 days in culture (Fig. 3A–C). Mesenchymal cells, which were labelled near to the tip of the bud, formed a crescent at the base of the developing tooth germ (Fig. 4) ($N = 10$). Sections clearly showed the labelled cells forming an arc underlying the dental papilla (Fig. 4D,E). In some cases the DiI labelled cells ended up on the opposite side of the tooth germ, indicating the dynamic kinetics of these cells (Fig. 5E).

Tooth germ cells contribute to alveolar bone formation

In slices cultured for 8 days, the formation of alveolar bone could be observed in histology sections. This bone formed around the bell stage tooth germ (Fig. 5C,F). DiI labelled cells, originating from the condensing mesenchyme (Fig. 5A,B), were found both surrounding and embedded within the developing bone (Fig. 5F–M). From their morphology and position within the bone, the labelled cells were shown to include osteocytes, rounded cells encapsulated in bone matrix, and osteoblasts, cuboidal cells lining the developing bone matrix (Fig. 5H–M).

DISCUSSION

DiI labelling of the condensing mesenchyme around the bud stage tooth germ shows the dynamic nature of these cells during tooth development. Cells moved from one side of the tooth germ to the other, spreading out in an arc under the developing papilla. A similar highly dynamic situation has recently been described in salivary glands, where the epithelium undergoes extensive re-arrangements during development (Larsen

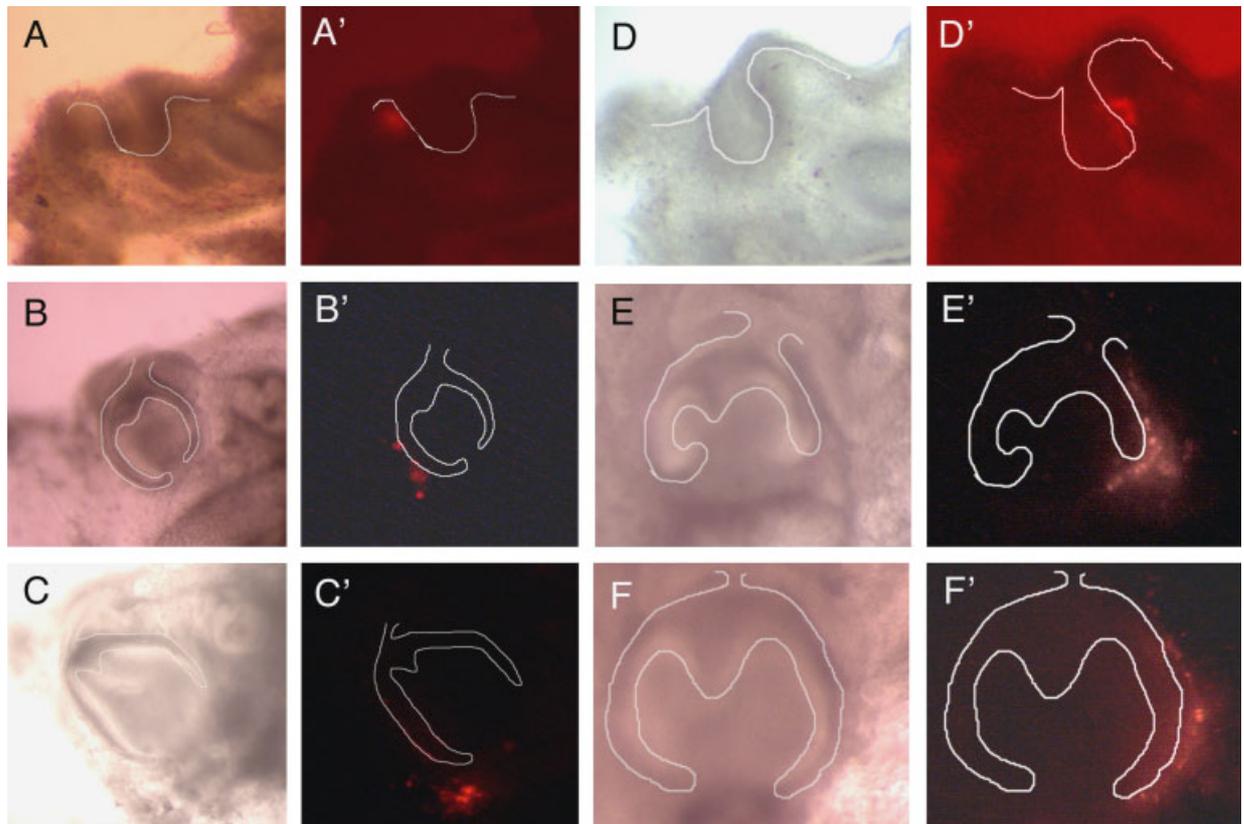


Fig. 3. Kinetics of mesenchymal cells toward the presumptive roots and periodontium. (A–C) Labelling of the mesenchymal cells on the buccal side of the tooth germ close to the border with the epithelium. Same culture on day 0 (A), day 4 (B), and day 6 (C). (A–C) Bright field images. (A'–C') Dark field images of slices A–C. Epithelium is outlined in white. Labelled cells appear red. (D–F) Labelling of the mesenchymal cells on the lingual side of the tooth germ close to the border with the epithelium. Same culture on day 0 (D), day 4 (E), and day 6. (D–F) Bright field images of slices. (D'–F') Dark field images of slices D–F. Epithelium is outlined in white and the DiI labelled cells appear red. Labelled cells closely follow the extending epithelium.

et al., 2006). Mesenchymal cells labelled at the top of the bud, close to the epithelium, remained in close contact with the epithelium and spread out in a line following the extension of the cervical loops. A movement from the oral surface toward the position of the future roots was observed in most cases.

Extensive cell movement of dental follicle cells has previously been shown by DiI labelling of postnatal day 6 first and second molars in vivo, and of dissected cap/early bell stage tooth germs in culture (Diekwisch, 2002). Follicle cells labelled with DiI were shown to move apically toward the roots at both stages. A general downward movement of mesenchymal cells, therefore, appears to occur throughout tooth development.

When condensing mesenchymal cells were labelled in our experiments, fluorescent cells were found in a crescent under the papilla, where the alveolar bone forms. This arc of cells mimics

expression pattern of genes such as *RANKL*, a mediator of bone metabolism (Ohazama et al., 2004). The curved arrangement of the DiI, thus, resembles a socket for the developing tooth. It was clear from sections that the osteocytes embedded in the developing alveolar bone were DiI positive. Thus, cells from the condensing mesenchyme around the epithelial tooth bud end up forming bone at the bell stage. The development of the tooth, and the bone into which it inserts, is therefore intricately linked by the same population of mesenchymal cells, allowing for the coordinated development of these two structures. A cellular continuity between tooth and alveolar bone formation may explain why alveolar bone formation fails to be initiated in mutants where tooth development is arrested at the bud stage, such as in the *Msx1*, *Pax9*, and *Lef1* mutants (Satakata and Maas '94; Van Genderen et al., '94; Peters et al., '98). In the *Msx1* and *Pax9* mutants

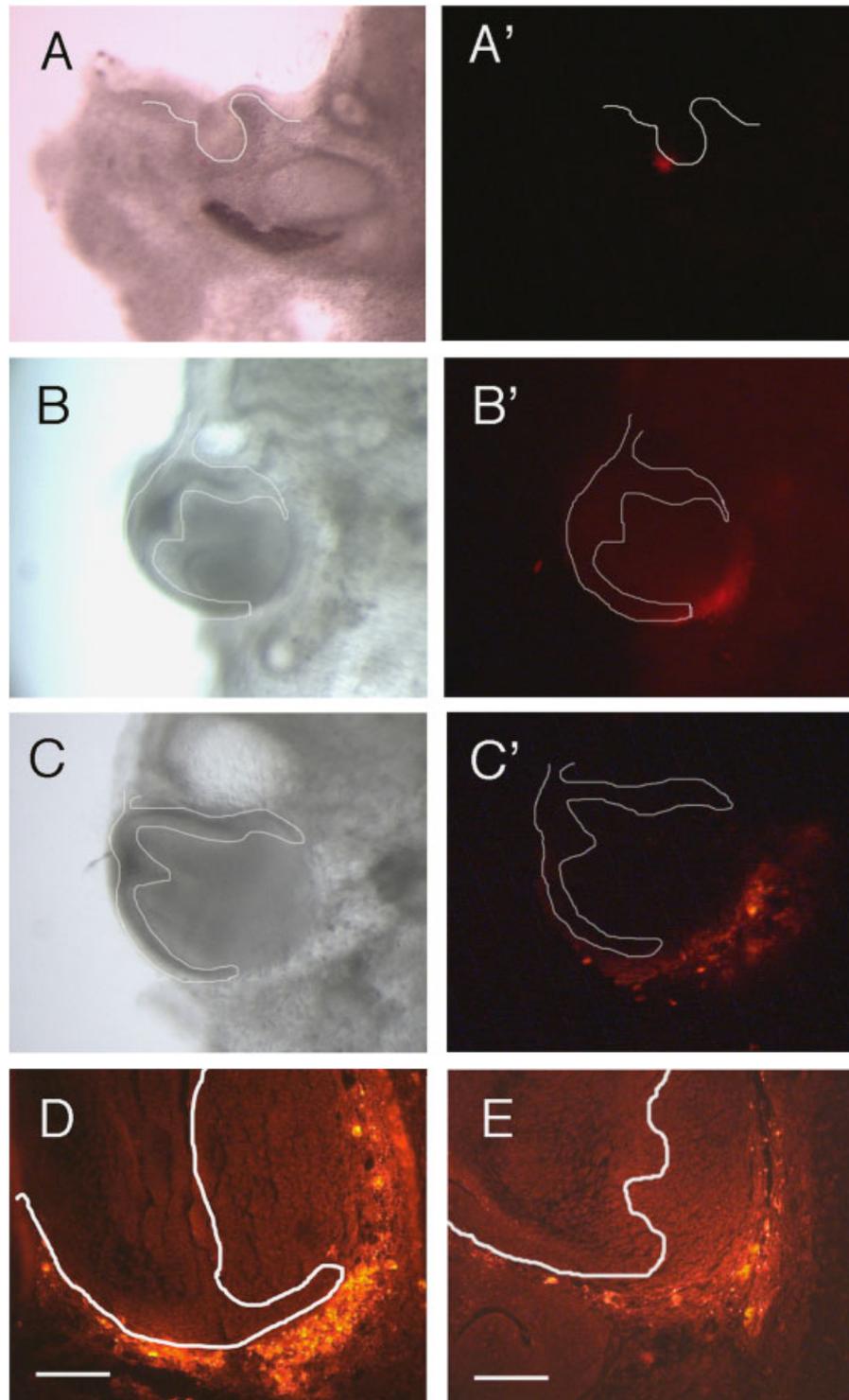


Fig. 4. Formation of a crescent of labelled cells under the dental papilla. (A–C) Labelling of the mesenchymal cells near the tip of the tooth germ close to the border with the epithelium. Same culture on day 0 (A), day 4 (B), and day 6 (C). (A–C) Bright field images of slices. (A'–C') Dark field images of slices A–C. (D, E) Sections through slices. Labelled cells lie in a crescent around the developing bell stage tooth germ. Epithelium is outlined in white. Scale bar in D and E = 100 μ m.

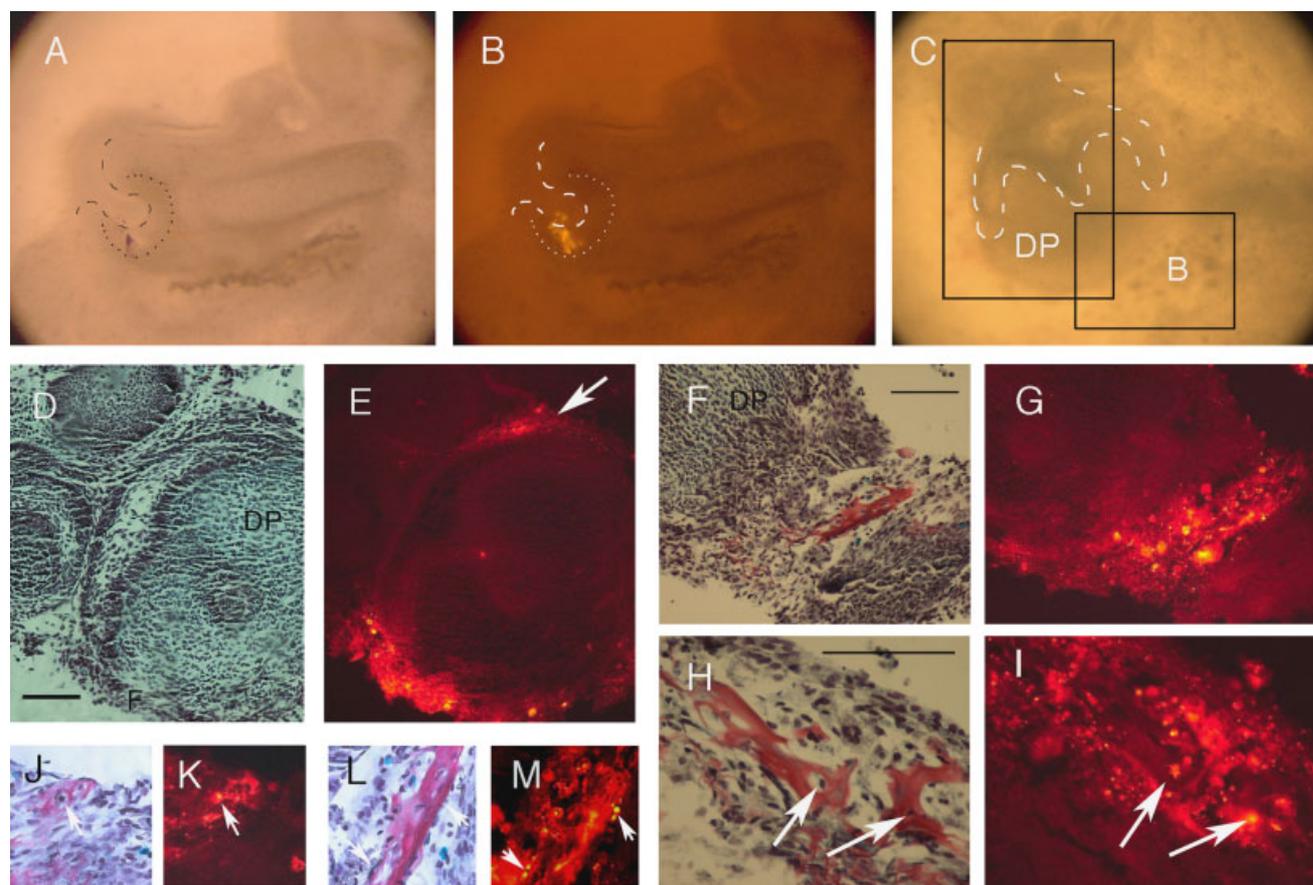


Fig. 5. Formation of alveolar bone by cells of the condensing mesenchyme at the bud stage. (A, C) Bright field. (B) Merged bright and dark field. (D, F, H, J, L) Sirius red/Alcian blue stain. (E, G, I, K, M) Dark field. (D–M) Sections through tooth germ shown in A–C. (A) Slice culture at E13.5. Day 0 (B) Labelling of the condensing mesenchyme on the buccal side of the tooth germ. A patch of epithelium of the bud is also labelled. The condensing mesenchyme and the epithelial bud are outlined in white. (C) Same slice after 8 days in culture, showing a bell stage tooth germ. Outlined in white. Boxes represent areas shown in section in (D) and (F). (D) Section of bell stage tooth germ. (E) DiI cells are now found surrounding the developing tooth in the investing layer of the follicle. Arrow indicates cells on the lingual side of the tooth. Label is also observed in the epithelium. (F) Basal part (apical) of tooth germ showing the developing alveolar bone. (G) The region of bone coincides with the DiI label (same section as F). (H–M) High magnification of forming alveolar bone. (H, J) Osteocytes embedded in the developing bone are arrowed. (I, K) Osteocytes (arrow) are labelled with DiI (same section as in H, J). (L) Cuboidally shaped osteoblasts line the developing bone matrix (arrows). (M) Osteoblasts are labelled with DiI (arrows). Scale bar in D, F, and H = 100 μ m. DP, dental papilla; B, bone; F, follicle.

there is a reduced number of condensing mesenchymal cells around the tooth bud, which would leave a small pool of cells able to go on to form both the tooth and supporting structures. In the *Msx1* and *Pax9* knockouts, *Bmp4* expression is downregulated in the dental mesenchyme. If *Bmp4* expression is upregulated in the *Msx1* mutant, using *Bmp4* driven by the *Msx1* promoter, the tooth and alveolar bone phenotype can be partially rescued, confirming the key role of *Bmp4* in controlling the formation of the condensing mesenchyme at the bud stage (Zhao et al., 2000). In contrast in the *Lef1* knockout the expression of *Bmp4* and *Msx1* are unaffected and the number of

condensing mesenchymal cells around the epithelial bud appear normal (Kratowil et al., '96). Given this it is interesting to note that in recombinations of *Lef1* mutant dental mesenchyme with wild type dental epithelium, alveolar bone was able to form in culture, indicating the potential of the mutant mesenchyme to form bone (Kratowil et al., '96). These experiments highlight the importance of generating a bud stage tooth germ, expressing *Bmp4* in the dental mesenchyme, in alveolar bone formation.

Knowledge of how a tooth normally forms may aid tooth tissue engineering projects, where it is essential to understand the relative relationship

of the epithelium, dental papilla, and alveolar bone, in order to recreate a tooth *in vitro*. These data may also help to understand clinical cases of oligodontia (missing teeth) and socket formation defects.

ACKNOWLEDGMENTS

We thank Agi Grigoriadis for critical reading of this manuscript and analysis of osteocytes and osteoblasts. The investigation of tooth–bone interaction in the Brno lab is supported by the Grant Agency of the Czech Republic (524/08/J032), and molecular odontogenesis by the Grant Agency of the Academy of Sciences, Czech Republic (B500450802). Research in the Brno lab runs under the IRP IAPG No. AVOZ 50450515.

LITERATURE CITED

- Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127:1671–1679.
- Cho MI, Garant PR. 2000. Development and general structure of the periodontium. *Periodontology* 24:9–27.
- Diekwisch TGH. 2002. Pathways and fate of migratory cells during late tooth organogenesis. *Connect Tissue Res* 43:245–256.
- D'Souza RN, Aberg T, Gaikwad J, Cavender A, Owen M, Karsenty G, Thesleff I. 1999. *Cbfa1* is required for epithelial–mesenchymal interactions regulating tooth development in mice. *Development* 126:2911–2920.
- Ducy P, Zhang R, Geoffrey V, Ridall AL, Karsenty G. 1997. *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell* 89:747–754.
- Kim J-Y, Cho S-W, Hwang H-J, Lee M-J, Lee J-M, Cai J, Choi S-H, Kim C-K, Jung H-S. 2007. Evidence for expansion-based temporal BMP4/NOGGIN interactions in specifying periodontium morphogenesis. *Cell Tissue Res* 330:123–132.
- Kratochwil K, Dull M, Farinas I, Galceran J, Grosschedl R. 1996. *Lef1* expression is activated by *Bmp4* and regulates inductive tissue interactions in tooth and hair development. *Genes Dev* 10:1382–1394.
- Larsen M, Wie C, Yamada KM. 2006. Cell and fibronectin dynamics during branching morphogenesis. *J Cell Sci* 119:3376–3384.
- Matalova E, Antonarakis GS, Sharpe PT, Tucker AS. 2005. Cell lineage of primary and secondary enamel knots. *Dev Dyn* 233:754–759.
- Ohazama A, Courtney J-M, Sharpe PT. 2004. *Opg*, *Rank* and *Rankl* in tooth development: co-ordination of odontogenesis and osteogenesis. *J Dent Res* 83:241–244.
- Palmer RM, Lumsden AGS. 1987. Development of periodontal ligament and alveolar bone in homografted recombinations of enamel organs and papillary, pulpal and follicular mesenchyme in the mouse. *Arch Oral Biol* 32:281–289.
- Peters H, Neubuser A, Kratochwil K, Balling R. 1998. *Pax9* deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* 12:2735–2747.
- Radlanski RJ, Mocker E, Rahlfs D. 1998. Computer-aided graphical reconstructions of the development of murine dental primordia and surrounding structures from day 12 until birth. *Eur J Oral Sci* 106:71–79.
- Satakata I, Maas R. 1994. *Msx1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* 6:348–356.
- Ten Cate AR, Mills C. 1972. The development of the periodontium: the origin of the alveolar bone. *Anat Rec* 170:365–380.
- Ten Cate AR, Milla C, Soloman G. 1971. The development of the periodontium. A transplantation and autoradiographic study. *Anat Rec* 170:365–379.
- Van Genderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, Bruhn L, Grosschedl R. 1994. Development of several organs that require inductive epithelial–mesenchymal interactions is impaired in *LEF-1* deficient mice. *Genes Dev* 8:2691–2703.
- Vainio S, Jalkanen M, Vaahtokari A, Sahlberg C, Mali M, Bernfield M, Thesleff I. 1991. Expression of *syndecan* gene is induced early, is transient, and correlates with changes in mesenchymal cell proliferation during tooth organogenesis. *Dev Biol* 147:322–333.
- Wilkinson DG. 1995. *In situ hybridisation*, a practical approach. Oxford, UK: IRL Press.
- Yamazaki H, Tsuneto M, Yoshino M, Yamamura K-I, Hayashi S-I. 2007. Potential of dental mesenchymal cells in developing teeth. *Stem Cells* 25:78–87.
- Yoshikawa DK, Kollar EJ. 1981. Recombination experiments on the odontogenic roles of mouse dental papilla and dental sac tissues in ocular grafts. *Arch Oral Biol* 26:303–307.
- Zhao X, Zhang Z, Soog Y, Zhang X, Zhang Y, Hu Y, Fromm SH, Chen Y. 2000. Transgenically ectopic expression of *Bmp4* to the *Msx1* mutant dental mesenchyme restores downstream gene expression but represses *Shh* and *Bmp2* in the enamel knot of wild type tooth germs. *Mech Dev* 99:29–38.
- Zhang Z, Song Y, Zhang X, Tang J, Chen J, Chen Y. 2003. *Msx1/Bmp4* genetic pathway regulates mammalian alveolar bone formation via induction of *Dlx5* and *Cbfa1*. *Mech Dev* 120:1469–1479.