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Contribution of mesoderm to the developing dental papilla

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ABSTRACT Teeth develop from epithelium and neural crest-derived mesenchyme via a series of reciprocal epithelial-mesenchymal interactions. The majority of the dental papilla of the tooth has been demonstrated to be of neural crest origin. However, non-neural crest cells have also been observed in this region from the bud stage of tooth development onwards. The number of these non-neural crest-derived cells rises as the dental papilla develops. However, their origin is unknown. We have followed migration of cells into the tooth *in vitro* using DiI to fate map regions surrounding the developing tooth. To identify the contribution of mesodermally-derived cells, we have utilised *Mesp1cre/R26R* transgenic reporter mice. We document that cells outside the early tooth primordium migrate into the developing dental papilla from the late cap stage of development. Here, we show that migrating cells are mesodermally-derived and create a network of endothelial cells, forming the blood vessels of the tooth. No cells of mesodermal origin were present in the condensed mesenchyme surrounding the dental epithelium until the cap stage of tooth development. Mesodermally-derived cells start invading the dental papilla at the late cap stage, providing the blood supply to the dental pulp. Endothelial cells are able to invade the developing dental papilla *in vitro* using the slice culture method. Understanding the origin and timing of migration of the mesodermally-derived cells is an important advance in our understanding of how a tooth develops and is particularly relevant to studies which aim to create bioengineered teeth.

KEY WORDS: *tooth, dental papilla, Mesp1cre, mesoderm, endothelial cells*

Introduction

Teeth develop from dental epithelium and neural crest-derived mesenchyme driven by reciprocal interactions. The dental epithelium invaginates into the surrounding dental mesenchyme to form a tooth bud. The dental epithelium starts to fold to produce a cap shape, with the mesenchymal dental papilla forming between the extending epithelial cervical loops. The dental papilla goes on to form the dental pulp at the centre of the tooth. The dental mesenchyme has been shown to be of neural crest cell origin, by using a *Wnt1cre/R26R* reporter mouse (Chai *et al*. 2000). However, these authors also reported the presence of some non-neural crest cells from the bud stage of tooth development onwards. As the tooth develops to the cap and bell stages, an increasing number of non-neural crest cells were observed in the dental papilla. This dynamic distribution of neural crest cell-derived tissue was attributed to differential proliferation of non-neural crest derived cells and/or possible apoptosis of neural crest cells (Chai *et al*. 2000). The number of non-neural crest cells

Abbreviations used in this paper: E, embryonic day; IDE, inner dental epithelium; M1, first lower molar; Mesp1cre/R26R, Cre-recombinase expressed under the control of the Mesp1 promoter as a transgene crossed with the ROSA26 conditional reporter (R26R); ODE, outer dental epithelium; P, postnatal; VEGF, vascular endothelial growth factor; Wnt1cre/R26R, cre-recombinase expressed under the control of the Wnt1 promoter as a transgene crossed with the ROSA26 conditional reporter (R26R); X-Gal, β substrate (bromo-chloro-indolyl-galactopyranoside).

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in the dental papilla can also increase as a result of immigration of cells. In support of this, when wild-type tooth germs were dissected at the cap stage and implanted into *LacZ (β-galactosidase)* or *GFP (Green Fluorescent Protein)* reporter host mice, an influx of host derived cells into the dental papilla was observed after a week to two weeks of development (*Cho et al*. 2003; Nait Lechguer *et al*. 2008).

During tooth development blood vessels and nerves move into the developing dental papilla (the prospective tooth pulp) to provide nutrients, oxygen and innervation. The non-neural crest derived cells in the early bell stage papilla might therefore represent endothelial or neuronal cell types. Nerves are found within the branchial (pharyngeal) arches at the earliest stages of tooth development but remain far from the tooth region itself (Mohamed and Atkinson 1983). At the cap stage a network of nerves forms under the tooth with fibres moving into the dental follicle which surrounds the tooth. Nerve fibres do not enter the papilla however, until cytodifferentiation is well underway, which for the first molar occurs a few days after birth (Mohamed and Atkinson 1983). The non-neural crest cells in the papilla at the bell stage are therefore unlikely to be due to the influx of neuronal cell types. Blood vessels, in contrast, are present in the papilla at a much earlier stage. In mouse embryo the endothelial cell markers are expressed in the central part of the future pulp from the early bell stage (E16.0) of the first lower molar (M1) (Nait Lechguer *et al*. 2008). There is thus a correlation between the expression of endothelial cell markers and the increase in non-neural crest cells in the papilla (Chai *et al*. 2000). It therefore seems likely that at least some of the non-neural crest derived cells in the dental papilla can be accounted for by an influx of endothelial cells.

In summary these data suggest that non-neural crest cells are present in the mouse dental mesenchyme at the bud stage (E13.5) and blood vessels are present in the dental papilla at the early bell stage (E16.0). However, it has not been proven whether the first capillary network rises de novo (vasculogenesis) or by an invasion of pre-existing capillaries (angiogenesis). We have therefore focused on the development of blood vessels in the dental tissue to verify the hypothesis that the cells of non-neural crest origin in the dental papilla are endothelial cells of mesodermal origin, which have migrated into the tooth.

The migration of cells into the dental papilla during tooth development was investigated using DiI to label the cells adjacent to the dental papilla *in vitro*. Previous studies have shown that no blood vessel-like structures could be detected in the dental pulp in tooth germs cultured *in vitro* (Nait Lechguer *et al*. 2008). This represents a serious problem for studying long-term tooth development *in vitro* as the health of the tooth germ is compromised. We therefore took advantage of the slice culture technique, in order to include the tissue that normally surrounds the tooth germ, thereby providing a more natural environment (Matalova *et al*. 2005; Cho *et al*. 2007; Diep *et al*. 2009).

To determine the nature of the non-neural crest cells in the dental papilla, we investigated the contribution of mesodermally-derived cells to the tooth using the *Mesp1cre/R26R* reporter mouse. *Mesp1* is an early marker of mesoderm expressed from the onset of gastrulation (Saga *et al*. 1996). The *Mesp1cre/R26R* mouse allows the contribution of cells of mesodermal origin to be followed at any stage of mouse development (Saga *et al*. 1999) and has previously been used to describe the contribution of mesoderm to craniofacial development (McBratney-Owen *et al*. 2008; Yoshida *et al*. 2008). However, the contribution of mesodermally-derived cells to tooth development has not yet been described. Finally we investigated the identity of the mesodermally-derived cells using immunohistochemistry.

Results

Movement of cells into the dental papilla in vitro

Tissue slices have been shown to provide a good method of visualising tooth germs as they develop *in vitro* (Matalova *et al*. 2005; Cho *et al*. 2007; Diep *et al*. 2009) (Fig. 1 A,B). Here we show that this culture method also allows vascularisation of the developing dental papilla. For this we used 250 μm slices of E14.5 mouse embryonic mandibles comprising the M1 tooth germ at the cap stage and cultured them for 4 days *in vitro*. To follow the blood vessel development during culture we used CD31 (PECAM-1) antibody as a specific endothelial marker (Newman *et al*. 1990). At the cap stage, the CD31 positive cells were present in the dental follicle surrounding the forming dental papilla (Fig. 1C). After 4 days in culture, the CD31 positive cells had entered the dental papilla, mimicking the expression observed *in vivo* (Fig. 1D) (Nait Lechguer *et al*. 2008).

To follow the fate of cells we labelled small groups of cells at the periphery of the condensed mesenchyme with a lipophilic dye – DiI (Diekwisch 2002; Diep *et al*. 2009) on the tissue slices as described above (Fig. 2A). No movement of the labelled cells into the dental papilla was observed over the first 24 hours in culture but after 2 to 3 days, as the tooth germ reached the late cap/early bell stage, DiI labelled cells were observed delaminating from the

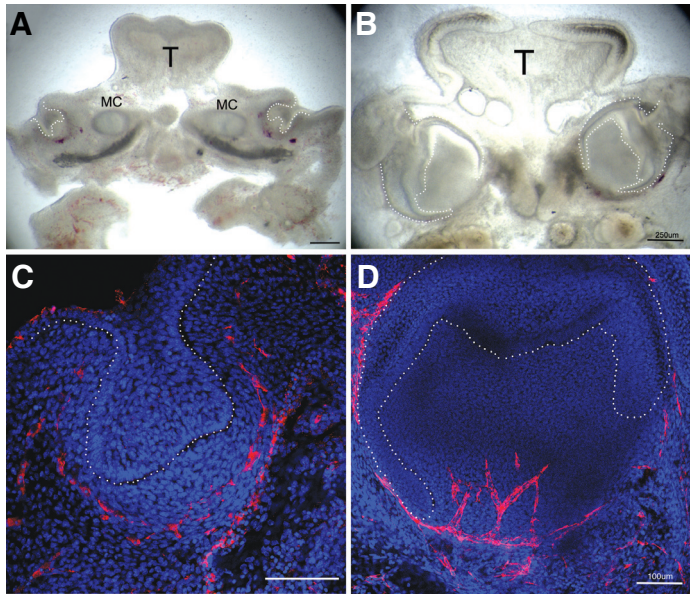

Fig. 1. Slice culture *in vitro* technique and the blood vessel invasion in vitro. (A) *Slice of the E14.5 mouse mandible.* **(B)** *The same slice after 4 days* in vitro *culture. MC, Meckel cartilage; T, Tongue.* **(C,D)** *Sliced molar tooth germs. Nuclei stained in blue with Hoechst, CD31 positive cells in red.* **(C)** *E14.5. At this stage there are no CD31 positive endothelial cells in the forming dental papilla.* **(D)** *Molar tooth germ after 4 days* in vitro *culture. The CD31 positive cells have invaded the dental papilla. The dental epithelium is highlighted by the white dotted line.*

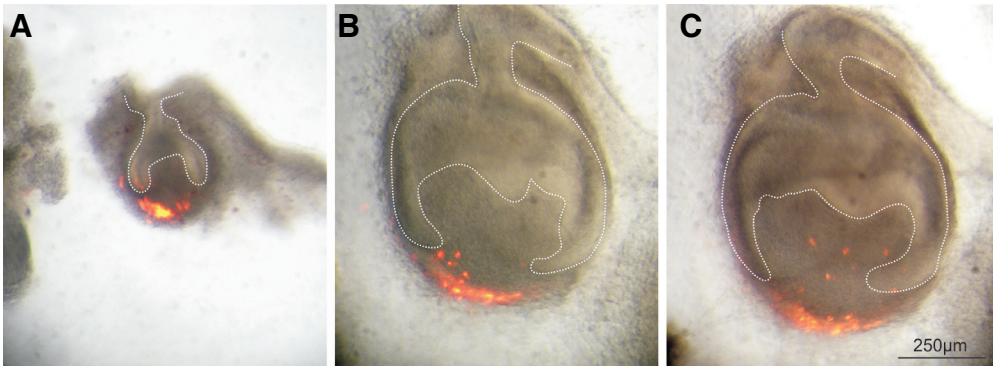

Fig. 2. Cell migration into the dental papilla. (A) *E14.5 molar tooth germ. Cells at the periphery of the condensed mesenchyme labelled in red with DiI.* **(B)** *The same molar tooth germ after 3 days* in vitro *culture. A few DiI labelled cells have separated from the main DiI labelled region and have started to migrate towards the dental papilla.* **(C)** *The same molar tooth germ after 4 days* in vitro *culture. The separated DiI labelled cells have migrated further into the dental papilla. The dental epithelium is highlighted by the white dotted line.*

main DiI labelled area (Fig. 2B). After 4 days in culture DiI labelled cells moved further into the developing dental papilla in a dispersed manner (Fig. 2C)(migration into dental papilla observed in N=48 cultures). This finding documents that some cells which contribute to the dental papilla at the bell stage are originally located in the periphery of the condensed dental mesenchyme at the cap stage.

Mesodermal cell contribution to the developing tooth

To analyze the origin of the DiI labelled cells migrating into the developing dental papilla *in vitro* we performed the same experiment as described above, using a *Mesp1cre/R26R* reporter mouse. The DiI labelled cells which invaded into the dental papilla over-lapped with the X-Gal labelled mesodermally-derived cells (Fig. 3 A,B). On histological sections it is clear that the DiI labelled cells are linked to the mesodermally-derived cells, with the DiI label located along the blue labelled tracts of the mesodermally-derived tissue (Fig. 3 C,D).

To trace the mesodermal contribution to the tooth germ *in vivo* we looked at histological sections of the *Mesp1cre/R26R* reporter mouse during various stages of tooth development. At the bud to cap transition stage there were no cells of mesodermal origin in the forming dental papilla. Mesodermally-derived cells were however located in the dental follicle (i.e. in the condensed mesenchyme adjacent to the papilla and outer dental epithelium – ODE – of the enamel organ) and in surrounding tissue (Fig. 4A). A few hours later at E15.0, a few blue cells were observed in the dental papilla as the tooth germ progressed to the late cap stage (Fig. 4B). At the early bell stage (E 16.5) mesodermally-derived cells were noted not only throughout the dental follicle but also in the dental papilla, reaching up to the odontoblast layer (Fig. 4C). At postnatal stage (P)5 mesodermally-derived cells were found scattered throughout the dental pulp (Fig. 4D). At P18, when the first molars have started to erupt and roots are formed, the blue stained mesodermally-derived cells marked out a crisscrossing network of cells in the dental pulp, with high concentrations located at the apex of the roots (Fig. 4E). Cells of mesodermal origin therefore enter the developing dental papilla at E15.0 and then spread throughout the forming dental pulp.

Endothelial cells in the developing tooth

The pattern of the mesodermally-derived cells at E14.5 and E16.5 (see Fig. 4 A,C) closely matched that of the endothelial cells labelled with the CD31 antibody (see Fig. 1 C,D and Fig. 5 A,B). In keeping with this, the *Mesp1cre/R26R* reporter has been shown to label endothelial cells which migrate throughout the head during development forming the blood vessel network (Yoshida *et al*. 2008). To confirm that the mesodermally-derived cells moving into the dental papilla were endothelial cells, CD31 immunohistochemistry was performed on *Mesp1cre/R26R* stained sections. A clear co-localisation of CD31 and mesodermally-derived cells was detected in the dental pulp (Fig. 5C), document-

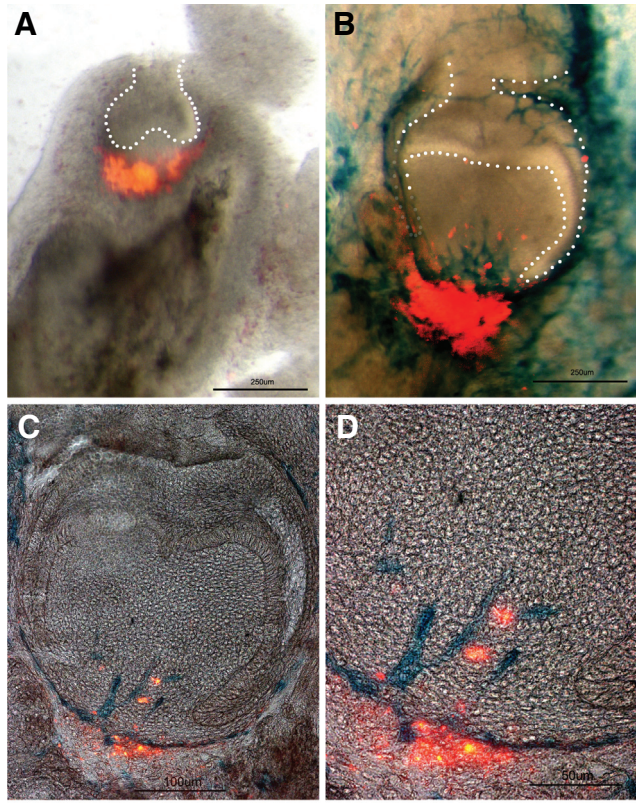

Fig. 3. Migration of mesodermally-derived cells into the dental papilla. (A) *E14.5 molar tooth germ of* Mesp1cre/R26R *embryo. Cells at the periphery of the condensed mesenchyme labelled in red with DiI.* **(B)** *The same molar tooth germ after 4 days in culture and X-Gal staining. The DiI labelled cells (in red) entered the dental papilla. The mesodermally-derived cells are stained in blue with X-Gal.* **(C)** *Histological section of the same X-Gal stained molar tooth germ. DiI in red, mesodermally-derived cells in blue.* **(D)** *Higher magnification of the same molar tooth germ showing the DiI labelled cells linked to the blue mesodermally-derived cells in the dental papilla.The dental epithelium is highlighted by the white dotted line.*

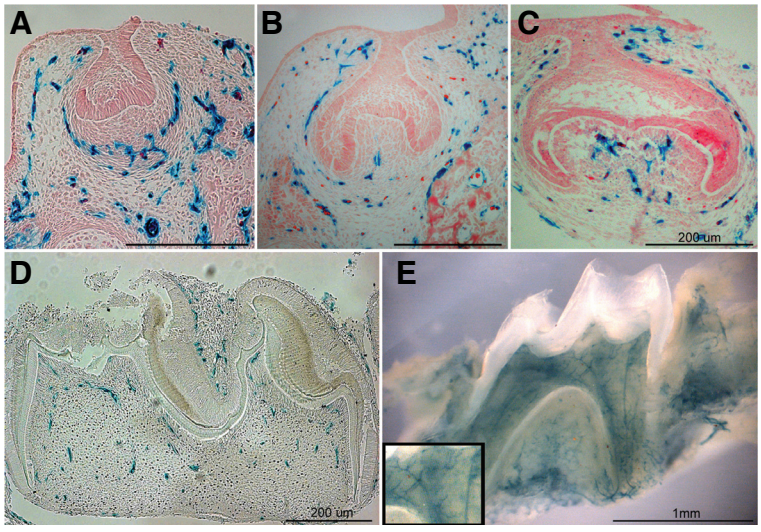

Fig. 4. Contribution of mesodermally-derived cells to molar development. *The mesodermally-derived cells are stained with X-gal in blue.* **(A)** *Frontal section of molar tooth germ of E14.5* Mesp1cre/R26R *embryo. There are no cells of mesodermal origin present in the dental epithelium or the adjacent condensed mesenchyme.* **(B)** *Frontal section of molar tooth germ of E15.0* Mesp1cre/R26R *embryo. Single mesodermally-derived cells are found in the tooth papilla.* **(C)** *Frontal section of molar tooth germ of E16.5* Mesp1cre/R26R *embryo. The mesodermally-derived cells are present in the dental papilla and the forming dental follicle.* **(D)** *Sagittal section of the first molar of* Mesp1cre/R26R *mouse at P5.* **(E)** *Sagittal view of the first molar of* Mesp1cre/R26R *mouse at P18, showing large numbers of mesodermally-derived cells in the dental pulp and at the roots with closer look at the capillary network in the frame.*

ing the immigration of endothelial cells of mesodermal origin into the dental papilla.

Discussion

By culturing the *Mesp1cre/R26R* tooth germs in slice culture we have shown that mesodermally-derived blood vessels were able to successfully invade the dental papilla *in vitro*. This finding is in contrast to the lack of blood vessel invasion observed in isolated tooth germs after *in vitro* culture (Nait Lechguer *et al.* 2008). The difference between these two *in vitro* culture methods probably lies in the amount of tissue left surrounding the tooth germ, which is a prerequisite for the blood capillaries to invade the dental papilla. This is supported by the fact that well-formed blood vessels invaded the papilla of dissected tooth germs after subcutaneous implantation, where the implanted tooth germ is surrounded by the host connective tissue rich in blood vessels (Nait Lechguer *et al.* 2008). These differences also suggest that the blood vessels in the dental papilla originate by angiogenesis (generation of vessels by sprouting from pre-existing capillaries) rather than vasculogenesis (de novo formation of a vascular network from endothelial precursors) (reviewed in Byrd and Grabel 2004).

The majority of the mesodermally-derived cells appear to be CD31 positive endothelial cells which move into the tooth to provide the blood supply. Endothelial cells have been shown to be a highly invasive cell population throughout the head (Yoshida *et al.* 2008). During the bell stage of tooth development, the endothelial cells rapidly colonise the dental papilla reaching up to the odontoblast layer. The close relationship between the odontoblasts and peripheral capillaries of the tooth has been related to the secretory activity of the odontoblasts (Yoshida and Ohshima 1996).

At the early cap stage a network of mesodermally-derived endothelial cells sits adjacent to the dental papilla but does not enter the papilla until the late cap stage. What controls the movement of the endothelial cells into the papilla? Blood vessels growth is regulated by the Vascular Endothelial Growth Factor (VEGF) (Mustonen and Alitalo 1995). Interestingly, one of the receptors for VEGF is Neuropilin, which was first discovered as a receptor for the semaphorin family of axon guidance molecules (reviewed in Geretti *et al.* 2008). Semaphorin-3a is expressed in the dental mesenchyme and has been shown to have a repulsive role: by defining where axons can migrate in the tooth by inhibiting axon growth into the regions where the sema3a is expressed (Loes *et al.* 2001). Semaphorin members may therefore act as guidance cues for both nerves and blood vessels. Other molecular cascades involved in angiogenesis include Notch and Hedgehog signalling which can target endothelial cells directly or can stimulate blood vessel support cells to produce VEGFs. For example, addition of Sonic hedgehog promotes expression of VEGF ligands (reviewed in Byrd and Grabel 2004). In the developing tooth, low levels of VEGF have been observed in the epithelium and dental papilla at the cap stage, with levels increasing in the inner dental

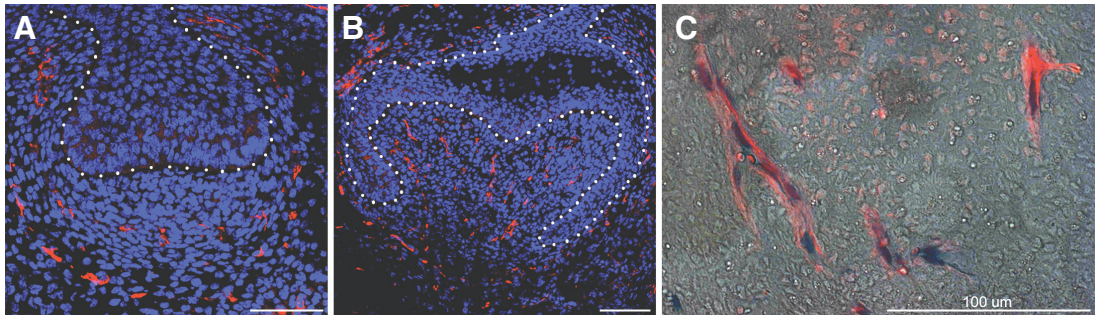

Fig. 5. Endothelial cells in the dental papilla. (A,B) *Frontal section of WT embryo at* **(A)** *E14.5* **(B)** *E16.5. CD31 positive endothelial cells are stained in red, nuclei with DAPI in blue. The CD31 pattern corresponds with the Mesp1-Cre/R26R X-gal stained molar tooth germ shown in Fig. 4.* **(C)** *CD31 staining on Mesp1-Cre/R26R X-gal stained sections of molar tooth germ at P5. CD31 positive endothelial cells are stained in red and are co-localized with X-gal stained blue mesodermally-derived cells.*

epithelium (IDE) at the bell stage (Aida *et al*. 2005). The increase in VEGF level coincides with the movement of endothelial cells into the papilla, and may play a stimulating role. The molecular mechanisms regulating blood vessels entering the dental papilla is an important question for future studies.

In conclusion, the increase of non-neural crest derived cells in the dental papilla during development is caused by the immigration of mesodermally-derived endothelial cells, which start to infiltrate the dental papilla at E15.0. No mesodermally-derived cells are present in the condensed mesenchyme before this time point. Our findings open an interesting question regarding the origin of the non-neural crest cells found at earlier stages of tooth development (Chai *et al*. 2000). From the *Wnt1cre/R26R* and *Mesp1cre/R26R* analysis these cells appear to be neither of neural crest nor of mesodermal origin. Thus either some other cell population contributes to the tooth mesenchyme at these early stages, or perhaps not all mesodermally or neural crest derived cells are labelled by the above-mentioned reporters.

Vascularisation of the tooth is an important process to ensure gas and metabolite exchange and therefore good vascularisation is an essential step for any attempts to bioengineer teeth (Nait Lechguer *et al*. 2008). Our study shows that migration of cells that will form the blood network of the tooth occurs relatively early during tooth development. Importantly blood vessel invasion can occur *in vitro* if the tissue surrounding the tooth germ is included in the explant. Knowledge of the timing and position of blood vessel precursors has a bearing on the health of tooth explants and the timing of implantation of bioengineered tooth germs. Our study also indicates that the *Mesp1cre/R26R* mice line could be an important tool in analysing the success of vascularisation in such studies.

Material and Methods

CD1 mice, Mesp1cre/R26R mice

All animals were killed using a schedule 1 method as approved by the Home Office and King's College London. The females were mated overnight and noon after the detection of the vaginal plug was considered as embryonic day (E) 0.5. For *in vitro* experiments and cryo-sections CD1 mice were used. The Mesp1cre heterozygous male was mated with homozygous Rosa26R female to generate the reporter line (Saga *et al*. 1999). Positive embryos were selected using a quick X-gal staining procedure on the embryonic bodies (20 minutes in X-Gal solution at 37°C).

Slice culture

E14.5 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 (Matalova *et al*. 2005; Diep *et al*. 2009). Slices showing a clear molar tooth cap were then selected and cultured. Slices were cultured on transparent nucleopore filters (VWR) supported on metal grids over medium. Medium consisted of DMEM F12 (Invitrogen) supplemented with 1% penicillin/streptomycin and 1% Glutamax (Invitrogen) and 10% fetal calf serum. Matrigel basement membrane matrix (7 μl) (BD Biosciences) was added on top of the slice. Slices were cultured at 37°C / 5% CO2 for 4-5 days.

DiI Labeling

DiI is a lipophilic dye, which intercalates in the cell membrane, marking groups of cells. DiI (Molecular probes cell tracker CM-DiI, C-7000) was dissolved in 100% EtOH. Small amounts of DiI were injected into the slices before culture using a mouth pipette (Diep *et al*. 2009). The slices were then placed on filters and cultured. The position of the DiI was recorded using fluorescent Leica dissecting microscope every 24 hours.

X-Gal staining

Mesp1-Cre/R26R positive embryo heads were dissected to remove the lower jaws at a range of stages (E14.5, E15.0, E16.5). Excess tissue was removed prior to staining. At postnatal stages (P5 and P18) it was necessary to remove the bone and the P18 molar was cut in half to allow the X-Gal staining solution to penetrate. The tooth germs were fixed in 4% PFA for 45 minutes, washed in PBS and stained in X-Gal staining solution for 42 hours at 37°C. The tooth germs were then post-fixed in 4% PFA O/N and then processed through a methanol series, isopropanol and tetrahydro-naphtalene to wax. Then the tooth germs were sectioned for 10 μm thickness for frontal and sagittal sections.

Immuno-fluorescent staining

For embryonic stages, the immuno-staining of CD31 antibody was performed on 10 μm thick frozen sections. The heads were dissected from CD1 mouse embryos at E14.5 and E16.5 and frozen immediately in O.C.T. Tissue Tek (Sakura) diluted 1:1 with Hank´s balanced solution (Sigma). Frozen sections were post-fixed in 4% PFA and processed by a Citric acid epitope retrieval method (boil in 10 mM citric acid pH 6.5). The sections were then incubated at 4°C O/N with primary antibody CD31 (Rabbit polyclonal IgG to CD31, Abcam, #Ab28364) diluted 1:150 in 1% BSA. The secondary antibody (Alexa Fluor® 568 goat anti-rabbit IgG, Invitrogen, #A11036) was diluted 1:500 in 1% BSA and incubated at RT for 1 hour. Nuclei were stained with Prolong® Gold anti-fade reagent with DAPI (Invitrogen).

For the postnatal stage P5, the Mesp1cre/R26R molars were embedded into wax and then sectioned for 5 μm thickness. The same staining protocol as described for frozen sections was then performed.

The slices of CD1 mandibles were fixed in 4% PFA 3hours at RT prior or after the *in vitro* culture, blocked in 10% goat serum in 0.1% Tween20 (Sigma) O/N. The slices were then incubated with CD31 primary antibody (1:150 in 1%BSA) for 6 hours, with the Alexa Fluor 568 (1:300 in 1%BSA) secondary antibody O/N and then stained with Hoechst (Sigma #H6024) for 4 hours. The images were taken using Leica SP5 confocal microscope.

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