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ABSTRACT

Teeth develop from reciprocal interactions between mesenchyme cells and epithelium, where the epithelium provides the instructive information for initiation. Based on these initial tissue interactions, we have replaced the mesenchyme cells with mesenchyme created by aggregation of cultured non-dental stem cells in mice. Recombinations between non-dental cell-derived mesenchyme and embryonic oral epithelium stimulate an odontogenic response in the stem cells. Embryonic stem cells, neural stem cells, and adult bone-marrow-derived cells all responded by expressing odontogenic genes. Transfer of recombinations into adult renal capsules resulted in the development of tooth structures and associated bone. Moreover, transfer of embryonic tooth primordia into the adult jaw resulted in development of tooth structures, showing that an embryonic primordium can develop in its adult environment. These results thus provide a significant advance toward the creation of artificial embryonic tooth primordia from cultured cells that can be used to replace missing teeth following transplantation into the adult mouth.

KEY WORDS: tooth development, bone-marrow-derived cells, stem cells, tissue engineering.

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INTRODUCTION

The application of stem-cell-based tissue-engineering approaches to create organs and tissues for transplantation requires an understanding and manipulation of the developmental processes that direct organ/tissue formation in the embryo, a source of cells with multipotential that can be easily cultured, and an ability of an organ rudiment to form the complete organ in the adult environment (Bianco and Robey, 2001). In common with most organs, teeth develop from interactions between epithelial cells (oral epithelium) and mesenchyme cells (cranial neural-crest-derived ectomesenchyme) (Thesleff *et al.*, 1995; Thesleff and Sharpe, 1997). Evidence accumulated from a variety of molecular and cellular studies has established that the embryonic oral epithelium provides the instructive signals for tooth initiation and shape determination. These signals principally consist of spatially restricted secreted protein ligands that are received by the ectomesenchyme cells which are then primed to become odontogenic and in turn act as a source of reciprocal signals back to the epithelium. In the early stages of jaw development (up to E10 in mice), all ectomesenchyme cells appear able to respond to signals such as FGF8 and BMP4 from the oral epithelium (Ferguson *et al.*, 2000). These observations, together with the ability of E10 embryonic oral epithelium to direct odontogenesis when recombined with non-dental ectomesenchyme, such as that from the second pharyngeal arch, suggest that ectomesenchyme cells are plastic in their responses to oral epithelial signals, and thus cranial neural crest cells do not contain an inherent odontogenic pre-specification (Mina and Kollar, 1987; Lumsden, 1988). These properties, together with the multipotentiality of cranial neural crest cells, prompted us to investigate the ability of cultured non-dental cells to replace ectomesenchyme cells and contribute to tooth formation.

MATERIALS & METHODS

Culture of Non-dental Cells

Feeder-independent mouse embryonic stem cells (E14.2) were cultured in D-MEM with 10^3 U/mL of leukemia inhibitory factor, buffalo rat liver cell-conditional medium, 200 mM L-glutamine, non-essential amino acid, and 2-mercaptoethanol. Medium was changed every day, and ES cells were passaged every 2-3 days. Duplicate flasks of the cells were used to generate a mouse gene knock-out that has subsequently resulted in two lines of mice with full germline transmission (unpublished).

Neural stem cells were isolated from E14 embryo spinal cords at the level of the upper limb to the lower cervical region. The cord itself was carefully dissected free from any other tissue and membrane to reveal nothing but naked spinal cord. The cord was then dissociated into single cells by the use of trypsin and flame-narrow pipettes and plated at 200,000 *per* T-75 on 10 μg/mL poly-ornithine and 10 μg/mL laminin in serum-free medium (DMEM/F12)

containing N2 neural supplement and 20 ng/mL FGF-2. Cells were cultured for 7 days before being harvested (Minger *et al.*, 1996). These tested 99% positive for neural stem cell marker nestin expression; their ability to differentiate into different neuronal cell types was assayed *in vitro*, and all three major neuronal cell types—neurons, oligodendrocytes, and astrocytes—were formed (see Appendix, Fig. 5).

Bone marrow cells were collected from tibiae and femora of 6- to 9-week-old female wild-type mice (CD-1). Five mice were killed by cervical dislocation, and tibiae and femora were aseptically removed and dissected free of adherent tissue. Both ends of the bone were cut, and the bone cavity was flushed out with culture medium slowly injected at the end of the bone by means of a sterile 21-gauge needle. Bone marrow stromal cells were subsequently suspended in α-minimal essential medium (Sigma) containing 20% heat-inactivated fetal bovine serum (FBS; Gibco BRL/Invitrogen, Paisley, UK) and 100 μM L-ascorbic acid 2-phosphate (Sigma) and were maintained for 10 days in a 75-cm^2 tissue flask. The medium was changed after 3 days and then subsequently every 2 days.

C3H10T1/2 and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) with 10% FBS. All solutions contained penicillin and streptomycin at 20 IU/mL.

Tissue Recombinations

To detect any possible contamination of embryonic oral epithelium with ectomesenchymal cells, we used transgenic mice expressing green fluorescent protein (GFP mice) as the source of the epithelium in the recombinations (Zambrowicz *et al.*, 1997; Hadjantonakis *et al.*, 1998). Following *in situ* hybridization, expression of *GFP* showed that non-dental cells in the recombinations were not contaminated with any ectomesenchyme cells. *In situ* hybridization for *GFP* expression on sections of teeth formed in renal capsules showed no expression in any mesenchyme-derived cells, whereas sections from teeth produced from *GFP* mice showed expression in all mesenchyme-derived cells (see Appendix, Fig. 6).

Mandible primordia of embryos (E10) from *GFP* mice were dissected in D-MEM with glutamax-1. The epithelium was isolated following incubation in a solution of Dispase (Gibco BRL) made up in calcium- and magnesium-free PBS at 2 U/mL for 10-15 min at 37°C. After incubation, the tissues were washed in D-MEM with 10% FBS, and the epithelium was mechanically separated by means of fine tungsten needles.

The cultured cell populations consisting of 5-6 x 10^6 cells were harvested by brief exposure to EDTA-Trypsin (2 g/L EDTA and 5 g/L Trypsin). After being washed several times, the cells were centrifuged to form a pellet which was then placed on transparent Nucleopore membrane filters (0.1 μm pore diameter; Coster) supported by a metal grid following the Trowell technique (1959) as modified by Saxén (1966). Three or four pieces of epithelium were then placed over the cell pellet and the recombinant explants incubated for 1-3 days at 37°C.

After the period of culture, the explants were fixed and processed for *in situ* hybridization or were transplanted under renal capsules. The explants were cultured in host kidneys for 10 days to allow for full development of teeth. The resulting tissues were then fixed and decalcified with the use of 0.5 M EDTA (pH 7.6).

In situ Hybridization

For *in situ* hybridization, explants were embedded and serially sectioned at 7-μm intervals. Sections were split over 5-10 slides. Radioactive *in situ* hybridization with the use of ^{35}S-UTP radiolabeled riboprobes was carried out according to previous reports (Angerer and Angerer, 1992; Tucker *et al.*, 1998). The mouse *Pax9* cDNA clone was a gift from Rudi Balling.

All experiments involving animals were carried out according to UK Home Office guidelines covered by Project and Personnel licenses to the corresponding author.

RESULTS

Three different sources of non-dental cells were assayed for their odontogenic responses to embryonic oral epithelium in these explant recombinations. Embryonic stem (ES) cells were used as a pure pluripotent stem cell population that would thus be expected to be able to form dental cells, given the appropriate signals. Neural stem cells were used as a pure multipotential stem cell population that is not known to be able to form dental cells. To assess the potential of an adult heterogeneous cell population to form teeth, we used bone-marrow-derived (BMD) cells. The ES cells were derived from the same passage of cells successfully used to generate germline chimeras. The neural stem cells were derived from a population that tested 99% positive for nestin expression (data not shown). The BMD cells were a mixed population shown to consist of fibroblasts, osteoblast and adipocyte progenitors, and up to 0.01% stem cells (Pereira *et al.*, 1998; Pittenger *et al.*, 1999). Cells were aggregated into a solid mass, overlaid with E10 oral epithelium, cultured *in vitro* for 3 days, and analyzed for expression of molecular markers of tooth development. Embryonic stem cells, embryonic neural stem cells, and adult BMD cells all responded identically by the induction of *Msx1*, *Lhx7*, and *Pax9* expression in a total of five recombinations *per* cell type (Figs. 1, 2A-2H). Although each of these genes is expressed in cells other than dental mesenchyme, the combination of expression of

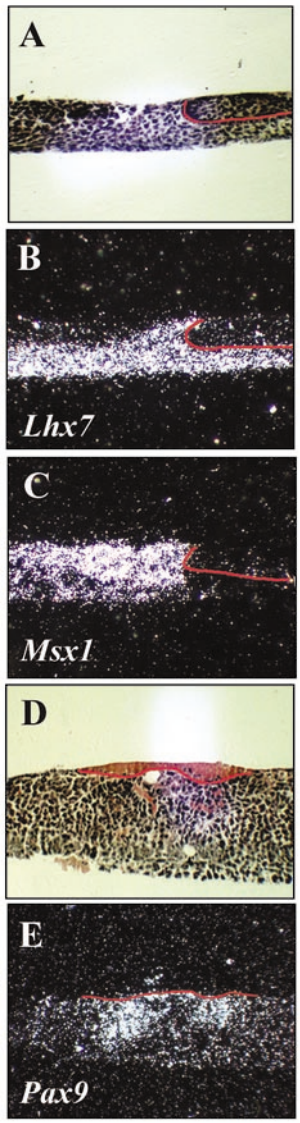

Figure 1. Sections of heterotypic recombinations between ES cell aggregations and embryonic oral epithelium. **(A,D)** Lightfield photomicrograph showing epithelial bud formation in recombinant explants. **(B,C)** Sections adjacent to (A) showing radioactive *in situ* hybridization for *Lhx7* (B) and *Msx1* (C). **(E)** Section adjacent to (D) showing radioactive *in situ* hybridization for *Pax9* (E). Tooth germ epithelium is outlined in red. Scale bar: 100 μm.

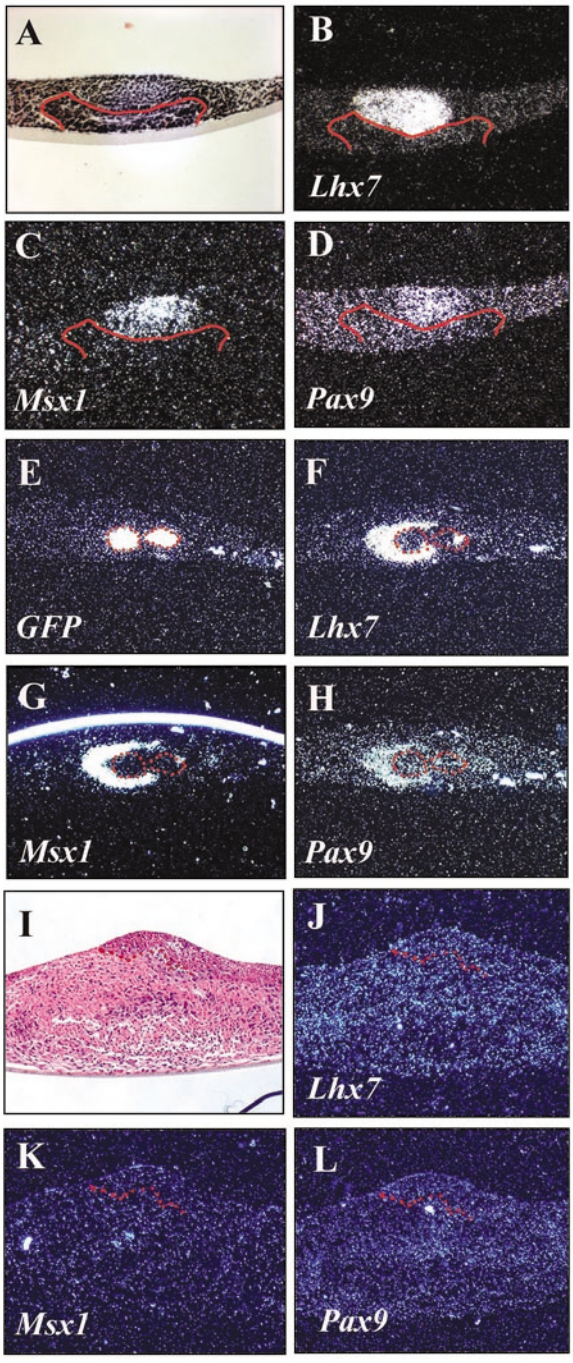

Figure 2. Sections of heterotypic recombination between neural stem cell aggregations and embryonic oral epithelium **(A-D),** between bone-marrow-derived cells and embryonic oral epithelium **(E-H),** and between NIH3T3 cell aggregations and embryonic oral epithelium **(I-L).** (A) Lightfield photomicrograph showing localization of epithelium in a recombinant explant. (B-D) Sections adjacent to (A) showing radioactive *in situ* hybridization for *Lhx7* (B), *Msx1* (C), and *Pax9* (D). (E-H) Adjacent sections of a recombination between bone-marrow-derived cells and embryonic oral epithelium. (E) Example of *GFP* expression in embryonic oral epithelium in a recombination. (F-H) Expression of *Lhx7* (F), *Msx1* (G), and *Pax9* (H) in bone-marrow-derived cells adjacent to the embryonic oral epithelium. (I) Lightfield photomicrograph showing localization of epithelium in recombinant explants. (J-L) Sections adjacent to (I) showing no expression of *Lhx7* (J), *Msx1* (K), and *Pax9* (L). Tooth germ epithelium is outlined in red. Scale bar: 100 μm.

these three genes is unique to odontogenic mesenchyme cells (MacKenzie *et al.*, 1992; Grigoriou *et al.*, 1998; Peters *et al.*, 1998). Recombinations were also carried out with cultured non-dental cell populations that are known not to have any multipotential stem-cell-like properties, such as NIH3T3 and murine mesenchymal cells (C3H10T1/2), and in these cases no expression of any of the marker genes was observed, while expression of non-odontogenic genes was found (Figs. 2I-2L; data not shown). Failure of tooth initiation in these control cultures showed that there was no contamination of the oral epithelium with ectomesenchyme cells. This was also confirmed by the use of genetically distinct oral epithelium from green fluorescent protein (*GFP*)-mice, where no expression was detected in non-dental mesenchyme cells in recombinations (Fig. 2E). The odontogenic response of the cultured non-dental 'mesenchyme' cell populations thus appears likely to be a stem cell property, but one that is not linked to tissue origin or developmental age.

To assess the ability of the non-dental cell explants to develop fully into teeth, we established recombinations between embryonic oral epithelium and aggregated non-dental cell populations from ES cells, neural stem cells, and BMD cells and transplanted them under the renal capsules of adult mice for 10-14 days. The resulting tissues that develop in the renal capsules are known to consist entirely of cells derived from the transplant. The host tissues make no cellular contribution to the donor tissues, and thus this procedure can be used to determine the extent to which recombinations containing non-dental cells can develop. In each of the three different types of recombinations, bone and soft tissue were formed from the explants, and in 3 explants from BMD cells, teeth were formed, surrounded by bone and soft tissue (Fig. 3; n = 35). The ES and neural cell stem recombinations were found to be very fragile and tended to dissociate when transferred to renal capsules. Formation of bone and soft tissue and failure to form intact teeth from mandibular primordia explants following such tissue disruption are consistent features of renal transfer. When this occurs, evidence of tooth tissue formation can sometimes be observed either histologically or with molecular markers. In several transplanted explants derived from ES and neural stem cells, expression of *Dspp*, a gene expressed predominantly in odontoblasts, was observed, indicating that even though intact teeth were not formed, tooth cell differentiation had occurred (see Appendix, Fig. 7). The BMD cells, however, formed a very robust explant, presumably due to the heterogeneous nature of the cell population. These explants could be easily transferred intact into renal capsules. The figure for the proportion of recombinations forming teeth was similar to the number we routinely obtain with manipulated recombinations between embryonic oral epithelium and ectomesenchyme, indicating that the non-dental cell mesenchyme behaves similarly to embryonic ectomesenchyme. Transplants of embryonic oral epithelium alone, stem cell and BMD cell aggregates alone, or recombinations between embryonic oral epithelium and NIH3T3 and C3H10T1/2 cells did not produce any bone or teeth. These control recombinations thus showed that there was no contamination of the oral epithelium with ectomesenchyme cells, or that the aggregated stem and stromal cells were not able to form teeth or bone in the absence of oral epithelium. Possible contamination by residual ectomesenchyme cells was

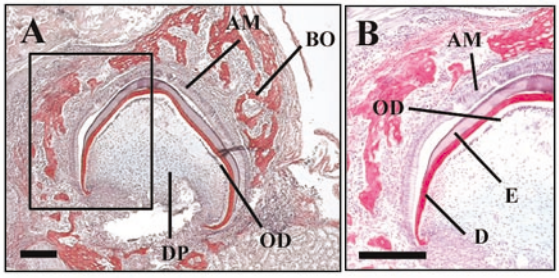

Figure 3. Recombinant explant between bone-marrow-derived cells and oral epithelium following 12 days of development in a renal capsule. All the tissues visible are donor-derived, since the host kidney makes no cellular contribution to the tissue. Where epithelium in the recombinations was from *GFP* mice, *in situ* hybridization of sections of these tissues confirmed that all mesenchyme-derived cells were of wildtype origin (not shown). BO, bone; Am, ameloblasts; DP, dental pulp; OD, odontoblasts, E, enamel; D, dentin. Scale bar: 80 μm.

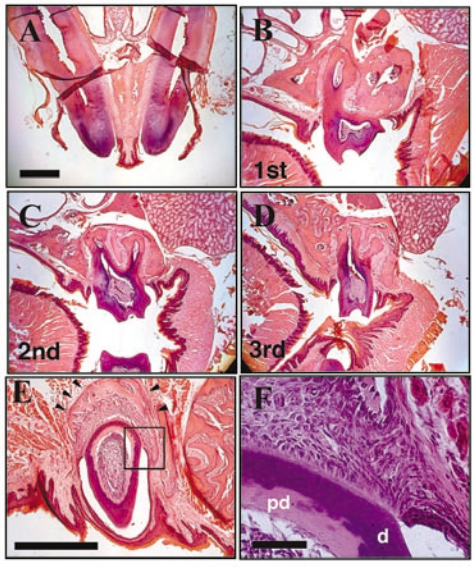

Figure 4. Upper jaw teeth in adult mouse mouth 26 days after transplantation of E14.5 molar explants. Molar tooth primordia with surrounding tissue dissected from E14.5 C56/B6 mice were cultured *in vitro* for 24 hrs. The mouse dentition consists of one incisor separated from three molars by a toothless region (diastema) in each quadrant of the mouth. A small incision was made in the soft tissue of the maxilla in the diastema of adult (over 20 wks) male mice. The explants (approximately 2 mm) were placed in the incision and fixed with surgical glue (Vetbond, 3M, St. Paul, MN, USA). The transplanted explants were left for 26 days, during which animals were fed a soft diet. After fixation and decalcification, wax serial frontal sections were cut and stained (H&E). **A**, incisors. **B**, first molar. **C**, second molar. **D**, third molar. **E**, ectopic tooth in diastema region (between A and B). Arrows show ectopic bone. **F**, High magnification of boxed area in E, showing periodontal-ligament-like tissue. d = dentin. pd = pre-dentin. Scale bars: 1.2 mm (A-D); 1.0 mm (E); 50 μm (F).

further eliminated by *in situ* hybridization that showed no *GFP* expression in any mesenchyme-derived cells (tooth, bone, or soft tissue) when epithelium was derived from *GFP* mice (see Appendix, Fig. 6).

The possibility that adult teeth can be formed from primordia derived *in vitro* from explants comprised of non-dental cultured cells is an important step in the tissue engineering of teeth for replacement. However, this approach relies on the ability of such embryonic primordia to develop into the complete organ when transferred to the adult, and whereas it is well-established that complete teeth can be functionally transplanted into the mouth, development of an embryonic primordium into a tooth in the adult jaw has never been shown (Andreasen *et al.*, 1990). Also, while this is possible in a few selected sites, such as the renal capsule and anterior chamber of the eye, there is no evidence, for any organ, that development can proceed normally at the appropriate adult site. To determine if a mouse embryonic tooth primordium could develop into a tooth when transplanted into the adult mouth, we surgically implanted E14.5 molar tooth rudiments into the soft tissue of the diastema of the maxilla of adult mice. The mouse dentition is comprised of one incisor separated from three molars by a toothless region (diastema) in each quadrant of the mouth. The transplanted explants were left for 26 days before fixation and decalcification for histology. Figs. 4A-4D show the normal histology of maxillary incisors (A) and molars (B-D). Fig. 4E is a section between the incisor (A) and molars (B-D) in the diastema, showing a clearly identifiable ectopic tooth formed at the site of the transplantation. The ectopic tooth was of a size similar to that of the first molar and was histologically normal, with dentin and enamel. The tooth was connected to ectopic bone by an organized soft connective tissue (Fig. 4F).

DISCUSSION

We show here that recombinations between mesenchyme created *in vitro* (by aggregation of non-dental cultured cells from different stem cell sources) and embryonic oral epithelium stimulate an odontogenic response in the mesenchyme, and that when such explants are transferred intact into adult renal capsules, they can develop into teeth (crowns) with associated bone and soft tissues. Thus, overall, analysis of these data shows that the odontogenic process can be initiated in non-dental cells of different origins, including purified stem cell populations and a mixed population of adult cells. Bone and soft tissues can be formed from non-dental cell populations consisting entirely of purified stem cells or from a heterogeneous population such as BMD cells. Tooth structures attached to bone could also be formed when intact explants such as those formed from BMD cells were transferred into renal capsules. BMD cells have recently been shown to be a convenient, non-pure source of stem cells that can form neurons following bone marrow transplantation in adult mice (Weimann *et al.*, 2003). The ability of this heterogeneous adult cell population to form bone and teeth in tissue-engineered rudiments is significant, since it implies that a pure population of stem cells is not necessary, and this may thus have important implications for the further development of these procedures in humans.

The embryonic oral epithelium is a simple, two-cell-thick ectoderm, and it is conceivable that this could be replaced with epithelial cells from another source. If this epithelium can be engineered to express the appropriate signals to initiate odontogenesis, a complete tooth primordium could be produced entirely from cultured cells. The identification of stem cells in

dental pulp and from exfoliated deciduous teeth also raises the possibility that a patient's own tooth cells could be used to generate new tooth primordia (Gronthos *et al.*, 2000; Miura *et al.*, 2003). The ability to tissue-engineer an organ rudiment such as a tooth primordium constitutes a major component of a regenerative medicine procedure (Chai and Slavkin, 2003). However, such organ primordia must be capable of developing into the complete organ *in situ*, in the appropriate site in the adult body. The renal capsule and anterior chamber of the eye are two adult sites that have been routinely used to support ectopic organ and tissue development, because they are immune-compromised and can provide an adequate blood supply to the transplanted tissue. To date, however, there have been no demonstrations of development of a complete organ at its normal location in the adult body following transplantation of an embryonic primordium. We show here that transfer of embryonic tooth primordia into the adult jaw resulted in complete tooth development, showing that an embryonic primordium can develop in its adult environment. The adult mouth is thus an appropriate environment to support development of an embryonic tooth primordium. Common abnormalities such as missing or damaged teeth are not life-threatening but are of obvious clinical importance. Although many important questions remain to be answered, such as the exact nature of the cells in the bone-marrow-derived population that form teeth, the provision of a suitable source of cells to replace embryonic oral epithelium—combined with the results presented here showing the ability of cultured, adult non-dental cells to replace embryonic mesenchyme cells in a tooth primordium, and the demonstration that an embryonic tooth primordium can develop into a complete tooth in the adult mouth—provides the real possibility that teeth could be produced 'to order' by the use of autologous adult non-dental cells to create tooth primordia *in vitro* for transplantation for replacement in humans. Moreover, the accessibility of teeth, together with the fact that they are not essential organs, mean that teeth provide an attractive organ with which to test the practicalities and feasibility of tissue-engineered organ replacement.

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REFERENCES

Andreasen JO, Paulsen HU, Yu Z, Ahlquist R, Beyer T, Schwartz O (1990). A long-term study of 370 autotransplanted premolars. Part 1. Surgical procedures and standardized techniques for monitoring healing. *Eur J Ortho* 12:3-13.

Angerer LM, Angerer RC (1992). In situ hybridisation to cellular RNA with radiolabelled RNA probes. In: In situ hybridisation: a practical approach. Wilkinson DG, editor. Oxford: Oxford University Press, pp. 15-30.

Bianco P, Robey PG (2001). Stem cells in tissue engineering. *Nature* 414:118-121.

Chai Y, Slavkin HC (2003). Prospects for tooth regeneration in the 21st century: a perspective. *Microsc Res Tech* 60:469-479.

Ferguson CA, Tucker AS, Sharpe PT (2000). Temporospatial cell interactions regulating mandibular and maxillary arch patterning. *Development* 127:403-412.

Grigoriou M, Tucker AS, Sharpe PT, Pachnis V (1998). Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* 125:2063-2074.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 97:13625-13630.

Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A (1998). Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech Dev* 76:79-90.

Lumsden AG (1988). Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* 103(Suppl):155-169.

MacKenzie A, Ferguson MW, Sharpe PT (1992). Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development* 115:403-420.

Mina M, Kollar EJ (1987). The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. *Arch Oral Biol* 32:123-127.

Minger SL, Fisher LJ, Ray J, Gage FH (1996). Long-term survival of transplanted basal forebrain cells following in vitro propagation with fibroblast growth factor-2. *Exp Neurol* 141:12-24.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, *et al.* (2003). SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 100:5807-5812.

Pereira RF, O'Hara MD, Laptev AV, Halford KW, Pollard MD, Class R, *et al.* (1998). Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci USA* 95:1142-1147.

Peters H, Neubüser 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.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al.* (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143-147.

Saxén L (1966). The effect of tetracyclin on osteogenesis in vitro. *J Exp Zool* 162:269-294.

Thesleff I, Sharpe P (1997). Signalling networks regulating dental development. *Mech Dev* 67:111-123.

Thesleff I, Vaahtokari A, Partanen AM (1995). Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. *Int J Dev Biol* 39:35-50.

Trowell OA (1959). The culture of mature organs in a synthetic medium. *Exp Cell Res* 16:118-147.

Tucker AS, Al Khamis A, Sharpe PT (1998). Interactions between Bmp-4 and Msx-1 act to restrict gene expression to odontogenic mesenchyme. *Dev Dyn* 212:533-539.

Weimann JM, Johansson CB, Trejo A, Blau HM (2003). Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. *Nat Cell Biol* 5:959-956.

Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci USA* 94:3789-3794.