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BRIEF COMMUNICATION

Chick Limbs With Mouse Teeth: An Effective In Vivo Culture System for Tooth Germ Development and Analysis

Eiki Koyama,* Changshan Wu, Tsuyoshi Shimo, and Maurizio Pacifici

Mouse tooth germ development is currently studied by three main approaches: in wild-type and mutant mouse lines, after transplantation of tooth germs to ectopic sites, and in organ culture. The in vivo approaches are the most physiological but do not provide accessibility to tooth germs for further experimental manipulation. Organ cultures, although readily accessible, do not sustain full tooth germ development and are appropriate for short-term analysis. Thus, we sought to establish a new approach that would combine experimental accessibility with sustained development. We implanted fragments of embryonic day 12 mouse embryo first branchial arch containing early bud stage tooth germs into the lateral mesenchyme of day 4–5 chick embryo wing buds in ovo. Eggs were reincubated, and implanted tissues were examined by histochemistry and in situ hybridization over time. The tooth germs underwent seemingly normal growth, differentiation, and morphogenesis. They reached the cap, bell, and crown stages in approximately 3, 6, and 10 days, respectively, mimicking in a striking manner native temporal patterns. To examine mechanisms regulating tooth germ development, we first implanted tooth germ fragments, microinjected them with neutralizing antibodies to the key signaling molecule *Sonic hedgehog* (Shh), and examined them over time. Tooth germ development was markedly delayed, as revealed by poor morphogenesis and lack of mature ameloblasts and odontoblasts displaying characteristic traits such as an elongated cell shape, nuclear relocalization, and amelogenin gene expression. These phenotypic changes began to be reversed upon further incubation. The data show that the limb bud represents an effective, experimentally accessible as well as economical system for growth and analysis of developing tooth germs. The inhibitory effects of Shh neutralizing antibody treatment are discussed in relation to roles of this signaling pathway proposed by this and other groups previously. *Developmental Dynamics 226:149–154, 2003.* © 2002 Wiley-Liss, Inc.

Key words: tooth development; organ culture; transplantation; chick limb bud; Sonic hedgehog; amelogenesis

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INTRODUCTION

The development of tooth germs continues to attract significant research interest because of the importance of normal dentition and the usefulness of these structures to analyze fundamental mechanisms of organogenesis (Lumsden, 1988; Slavkin and Diekwisch, 1996; Maas and Bei, 1997; Thesleff and Mikkola, 2002). Odontogenesis initiates with formation of dental laminas along the oral epithelium. Lamina cells then proliferate and migrate into the underlying ectomesenchyme and give rise to cap-stage tooth buds that display an inner dental epithelium, an outer dental epithelium, and a mesenchymal dental papilla. With further development, the tooth germs grow and progress through the bell stages, during which epithelial stellate reticulum and stratum intermedium form and dental cusps

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become well recognizable. Terminal cytodifferentiation leads to emergence of ameloblasts and odontoblasts and deposition of enamel- and dentin-rich extracellular matrices. Initiation and progression of tooth germ development require extensive and reciprocal interactions between epithelial and mesenchymal cells. Clearly, tooth germ development is a very complex process that involves time and the space-restricted processes of cell determination, proliferation, and migration, tissue-tissue interactions, morphogenetic movements, apoptosis, and cytodifferentiation.

There has been considerable progress in recent years in identifying mechanisms that regulate tooth germ development, but we are far from having a complete description and understanding of them (Thesleff and Sharpe, 1997). These advances have largely resulted from the use of three experimental approaches: in vivo analysis of tooth germs in normal and mutant mice; transplantation of tooth germs at ectopic sites of host animals; and organ culture. Each of these systems has advantages and disadvantages. The first approach is obviously the most physiological and quite powerful but is hampered by the difficulty of performing additional experimental interventions and manipulations. Transplantation of tooth germs to ectopic sites has been used widely; these sites include anterior chamber of the eyes (Kollar and Fisher, 1980; Yoshikawa and Kollar, 1981; Lumsden and Buchanan, 1986), renal subcapsular site (Bartlett and Reade, 1973), subcutaneous tissue (Ivanyi, 1965), cheek pouch (Al-Talabani and Smith, 1979), and spleen (Ishizeki et al., 1987). This approach has been particularly useful for tissue recombination experiments and analysis of tissue interactions. Its main limitation is that further experimental manipulations would require sequential rounds of surgery. Lastly, standard organ cultures of tooth germs or their fragments are popular because they are easy and experimentally accessible and have indeed led to many insights into the nature of tissue interactions and their roles in gene expression (Vainio et al., 1993). The major limitation is that the cultures can usually be maintained for relatively short periods of time to avoid tissue degeneration and that tooth germs are not able to progress from very early to late terminal stages of differentiation and organogenesis.

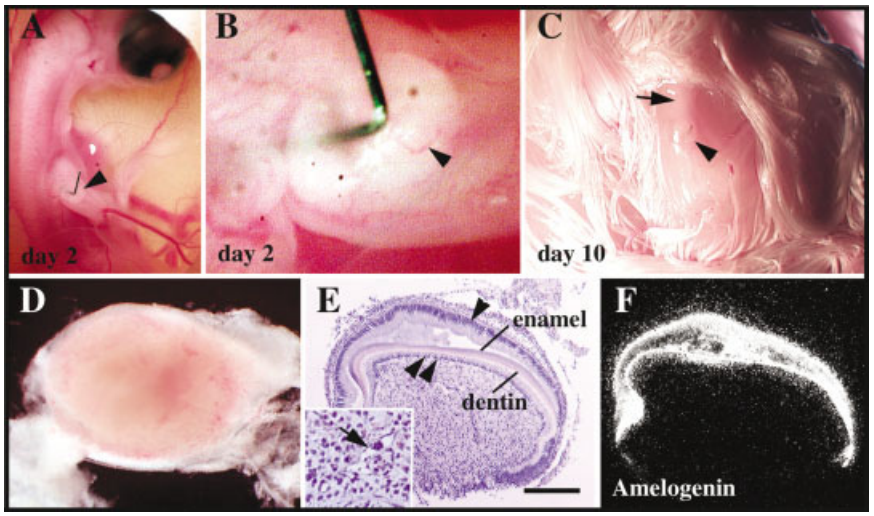

Fig. 1. Stereologic and histologic analysis of a embryonic day 14 tooth germ fragment implanted in host chick embryo. **A:** Wing bud on day 2 viewed through the open egg shell and bearing the dental tissue held by a tungsten wire (arrowhead). **B:** High magnification of specimen in A; arrowhead points to a neighboring blood vessel. **C:** Wing on day 10 of transplantation displaying a very large tissue mass (arrowhead) near the humerus (arrow). Note lack of feathers, which is likely due to removal of ectodermal tissue before transplantation. **D:** Anatomic view of tissue mass after dissection from host wing. **E:** Histology of tissue mass revealing the presence of a well-formed second molar tooth germ at crown stage. Arrowhead and double arrowhead point to ameloblasts and odontoblasts, respectively, with nuclei in their characteristic basolateral location. Inset shows a large vessel (arrow) within the dental mesenchyme. **F:** Darkfield image showing strong amelogenin gene expression. Scale bar = 250 μm in E (applies to D–F).

Given the above, it would be highly desirable to have a system that combines experimental accessibility with the ability to sustain full tooth germ development. In a previous study, we had implanted fragments of mouse tooth germs into chick embryo wing buds to analyze expression and function of the signaling molecule *Sonic hedgehog* (Shh; Koyama et al., 1996). Others transplanted embryonic mesenchymal tissues from facial primordia into chick limbs and found that they underwent substantial morphogenesis (Richman and Tickle, 1989). Thus, we asked in the present study whether the chick limb bud may offer a system for long-term growth and development of mouse embryo tooth germs and for analysis of mechanisms of regulation.

RESULTS AND DISCUSSION

We first determined whether mouse embryo tooth germs grow and develop within the environment of a chick limb. E14 mouse embryo mandibular fragments containing bud- and cap-stage molar tooth germs were transplanted onto the anterolateral mesenchyme of day 4–5 chick wing buds (one fragment/wing bud) and held in place with a tungsten wire (Fig. 1A, arrowhead). Macroscopic examination on day 2 showed that the transplanted tissue was easily observable and recognizable through the window in the eggshell and had remained in place (Fig. 1A). Large blood vessels were evident near the transplanted tissue (Fig. 1B, arrowhead), possibly representing the host's angiogenic response. Implanted tissues remained easily visible for up to 5 days from transplantation, after which detection became more difficult because the embryo is repositioned to the middle of the egg and the chorioallantoic membrane becomes

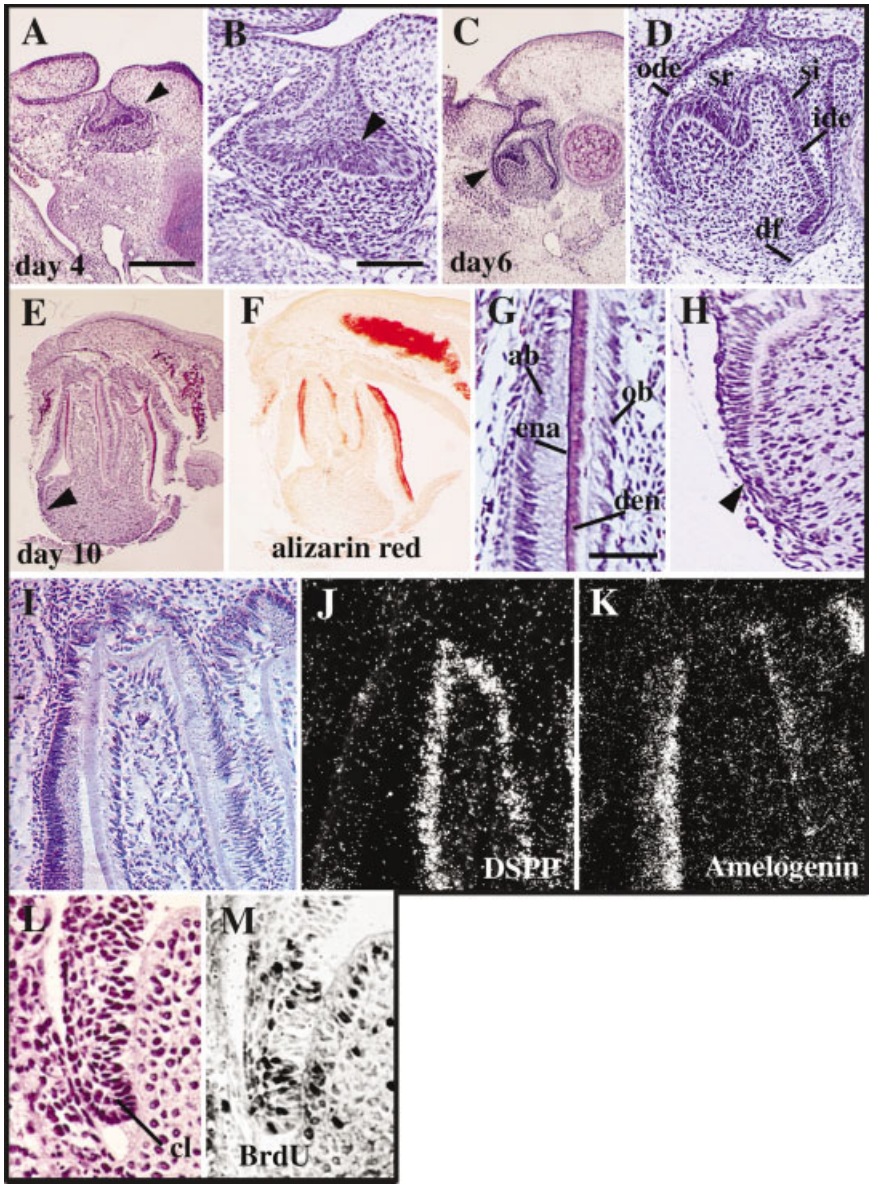

Fig. 2. Histologic, histochemical, and in situ hybridization analyses of transplanted embryonic day 12 tooth germs over time in ovo. **A:** Cap stage tooth germ (arrowhead) on day 4. **B:** Higher magnification of tooth germ in A showing a well-formed enamel knot (arrowhead). **C:** Early bell tooth germ (arrowhead) on day 6. **D:** High magnification of tooth germ in C with characteristic outer dental epithelium (ode), stellate reticulum (sr), stratum intermedium (si), inner dental epithelium (ide), and dental follicle (df). **E,F:** Crown stage tooth germ on day 10. Arrowhead in E points to developing epithelial root sheath. Alizarin red staining in F reveals distribution of mineralized matrices. **G,H:** Higher magnification views of crown stage germ showing enamel (ena), dentin (den), elongated ameloblasts (ab), and odontoblasts (ob) in G and epithelial root sheath in H (arrowhead). **I–K:** Companion crown stage sections (I) processed for in situ hybridization to reveal with gene expression of dentin sialophosphoprotein (J) and amelogenin (K). **L,M:** Sections from early bell stage tooth germ on day 6 after pulse-labeling with bromo-5′-deoxyuridine (BrdU) and immunohistochemical detection of incorporated BrdU. Note numerous dark-staining labeled proliferating epithelial and mesenchymal cells in and around the developing cervical loop (cl). Scale bars = 650 μm in A (applies to A,C,E,F), 200 μm in B (applies to B,D), 50 μm in G (applies to G–M).

thicker. By day 10 after transplantation, the implanted site exhibited a large tissue mass measuring 4 to 5 mm in length (Fig. 1C, arrowhead) and protruding away from the underlying host skeletal element (humerus) (Fig. 1C, arrow). Feathers, which otherwise covered much of the limb at this stage, were actually absent at the transplanted site (Fig. 1C).

The large tissue masses present at day 10 of transplantation were isolated (Fig. 1D) and processed for histology and in situ hybridization. Quite well-formed first and second molar tooth germs at crown stage were present and contained terminally differentiated ameloblasts and odontoblasts (Fig. 1E). Both cell types displayed characteristic morphologies and organization and had deposited an enamel- and dentin-rich matrix covering the entire cusp (Fig. 1E). The ameloblasts contained high levels of RNA encoding amelogenin, a characteristic gene product (Fig. 1F), and the nuclei of ameloblasts and odontoblasts were located near the basolateral membrane (Fig. 1E, arrowhead and double arrowhead, respectively), which is a cytologic feature associated with their terminal differentiation (Koyama et al., 2001). Numerous blood vessels were present in the dental papilla (Fig. 1E inset, arrow). Over 20 independent sets of transplantations produced comparable results.

To monitor the progression of tooth germ development, early bud stage tooth germs from embryonic day (E) 12 mouse embryos were transplanted into day 4–5 limb buds and examined at several time points. By day 4 of transplantation, the tooth germs had reached the cap stage (10 of 10; Fig. 2A, arrowhead) and the enamel knot was forming in the center of the enamel organ (Fig. 2B, arrowhead). By day 6, the tooth germs had advanced to the early bell stage (6 of 6; Fig. 2C, arrowhead) in which outer dental epithelium, stellate reticulum, stratum intermedium, inner dental epithelium, and dental follicle were all apparent and well formed (Fig. 2D). By day 10, the tooth germs were at the crown stage (8 of 9; Fig. 2E). They contained mineralized alizarin red–positive matrix (Fig. 2F), abundant enamel and dentin (Fig. 2G), an epithelial root sheath in the cervical region (Fig. 2E,H, arrowhead), and strong expression of such terminal gene markers as dentin sialophosphoprotein (DSPP) and amelogenin (Fig. 2I–K).

Tooth germ development in-

volves restricted patterns of cell proliferation (Coin et al., 1999). To determine whether those patterns were reproduced and could be readily analyzed in limb-implanted tooth germs, bromo-5′-deoxyuridine (BrdU) was injected into the chick host; 2 hr later, limbs were processed for immunohistochemistry. Indeed, BrdU-labeled proliferating cells were clearly apparent at numerous and appropriate locations (not shown). For example, there were many labeled epithelial cells in the cervical loop and neighboring mesenchymal cells in early bell specimens on day 6 of transplantation (Figs. 2L,M), closely resembling endogenous proliferation patterns seen in mouse embryos (Casasco et al., 1995).

In the next set of experiments, we examined mechanisms regulating development of transplanted tooth germs, focusing specifically on Shh, which is a signaling factor involved in and required for tooth germ development (Hardcastle et al., 1998; Dassule et al., 2000; Gritli-Linde et al., 2001; Koyama et al., 2001). Accordingly, E17 middle bell stage molar tooth germ fragments containing mesial cusps were transplanted onto host chick limb buds and were then treated with Shh-neutralizing antibodies (Marti et al., 1995). Different amounts of neutralizing antibodies or equal amounts of preimmune rabbit immunoglobulin (Ig) Gs (contained in a total volume of 1 μl) were microinjected in the vicinity of the tissues before transplantation and again after 24 and 48 hr. Specimens were examined on days 3 and 4 by histology and in situ hybridization. Control tooth germs on day 3 had advanced to the crown stage; they displayed terminally differentiated ameloblasts (ab) and odontoblasts (ob) with a typical elongated cell morphology and basal nuclear localization (Fig. 3A inset, arrowheads) and abundant amelogenin transcripts (7 of 7; Fig. 3B). In contrast, tooth germs treated with neutralizing antibodies displayed clear signs of growth and developmental retardation (Fig. 3C–F). Germs treated with 0.5 μg antibodies/injection were significantly smaller and still at the bell stage (8 of 8; Fig. 3E). They contained poorly differentiated cells that were not elongated and in which the nuclei were not located at a basolateral position (Fig. 3E inset, arrowheads). Amelogenin transcripts were completely absent (6 of 8; Fig. 3F). Both the cytologic and molecular traits of the cells indicated that they were still preameloblasts and preodontoblasts (Fig. 3E). Tooth germs treated with a lower dose of antibodies (0.1 μg/injection) were affected to a lesser degree, the major effect being an overall reduction of growth (2 of 7; Fig. 3C,D). When tooth germs were examined on day 4, they all displayed signs of recovery from neutralizing antibody treatment (5 of 5). There were elongating ameloblasts and odontoblasts at the cusps (Fig. 3G) as well as moderate amounts of amelogenin transcripts (Fig. 3H).

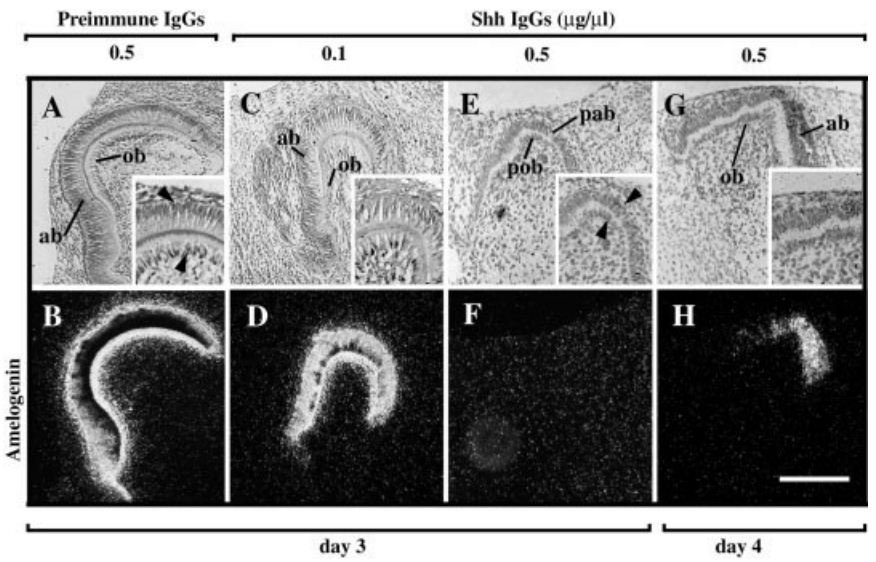

Fig. 3. Effects of neutralizing *Sonic hedgehog* (Shh) antibody treatment on tooth germ development in ovo. Embryonic day 17 bell stage mesial tooth germ fragments microinjected with 0.1 (C,D) or 0.5 μg (E–H) of neutralizing antibodies or 0.5 μg of preimmune immunoglobulin Gs (A,B) after isolation and again on day 1 and 2 after transplantation. **A,B:** Control crown stage tooth germs on day 3 showing terminally differentiated ameloblasts (ab) and odontoblasts (ob) (A) and very strong amelogenin gene expression (B). Inset in A shows basolateral nuclear localization (arrowheads). **C,D:** Crown stage tooth germ on day 3 exhibiting a fairly normal organization and gene expression but an overall reduced size. Inset in C shows no major effect on cell appearance. **E,F:** Tooth germ treated with 0.5 μg neutralizing antibody still at the bell stage and containing preameloblasts (pab), preodontoblasts (pob; E), and no detectable amelogenin transcripts (F). Inset in E shows cuspal cells (arrowheads) which are not elongated and do not have nuclei in a basolateral position. **G,H:** Tooth germ on day 4 treated with 0.5 μg of neutralizing antibody and exhibiting a few differentiated cuspal ameloblasts and odontoblasts (G), recovery of elongated cell architecture (inset in G), and detectable amelogenin transcripts (H). Scale bar = 250 μm in H (applies to A–H).

The results of the study show that the chick limb bud offers a suitable environment for mouse tooth germ development. The transplanted early bud, cap, or bell stage tooth germs all resume their development and progress to terminal stages of morphogenesis and cytodifferentiation. Early bud stage germs reach the cap, early bell, and crown stages in approximately 4, 6, and 10 days from transplantation, a time course remarkably similar to that seen in mouse embryos. As indicated by BrdU immunohistochemistry, the transplanted tooth germs possess characteristic patterns of cell proliferation that are in accord with their apparently normal growth and morphogenesis. The crown stage of odontogenesis is characterized by complex cell and tissue architectures and highly specialized functions, including expression of unique genes, deposition of enamel- and dentin-rich matrices that are then mineralized, and cytologic reconfiguration of the cells such as nuclear relocalization in ameloblasts and odontoblasts (Koyama et al., 2001). All these fea-

tures are displayed by crown stage tooth germs forming in the chick limb. Clearly, the limb is able to sustain a seemingly normal odontogenesis process even when the starting material is an early bud stage tooth germ. Thus, the chick embryo limb represents a new, powerful, effective, and economical addition to the methodologies currently used in tooth germ research.

We find that treatment with Shh-neutralizing antibodies delays growth and development of transplanted tissues. In comparison to controls, the day 3 treated tooth germs fail to reach the crown stage and lack terminally differentiated ameloblasts and odontoblasts displaying an elongated cell shape, nuclear relocalization, and strong amelogenin expression. These severe effects began to dissipate with further in ovo time with the emergence of differentiated cells at the cusps. We and others showed previously that, although Shh transcripts are limited to inner dental epithelium and preameloblasts, Shh itself is present also in the underlying mesenchymal tissues (Koyama et al., 1996; Gritli-Linde et al., 2001). This finding led us to propose that Shh may be important for differentiation of both ameloblasts and odontoblasts. This possibility is in line with the fact that Shh receptors are expressed by both epithelial and mesenchymal dental cell layers (Motoyama et al., 1998; Gritli-Linde et al., 2001; Koyama et al., 2001) and with our observation here that differentiation of both cell types is severely delayed by Shh-neutralizing antibody treatment. However, others have proposed recently that, although Shh has important roles in tooth germ development, it may not be required for cytodifferentiation. This conclusion was reached with mouse embryos in which the Shh gene was selectively ablated in tooth germs (Dassule et al., 2000). Compared with control, the Shh-null tooth germs were much smaller and not well organized but did display a few amelogenin-expressing ameloblasts at the cusps by E18.5, similar to those we see on day 4 in ovo. What do the results of that study and our study say about the roles of Shh in tooth germ development? Clearly, both studies demonstrate that Shh has several important roles in tooth germ development, one being control of overall growth. The presence of a few differentiated ameloblasts and odontoblasts in E18.5 Shh-null or day 4 antibody-treated tooth germs may signify that Shh is indeed not needed for dental cell differentiation (Dassule et al., 2000). On the other hand, the complete lack of differentiated cells in day 3 antibody-treated tooth germs (compared with the abundance of such cells in companion control specimens) could indicate that Shh is needed for differentiation. Compensatory mechanisms present in the tooth germs themselves or provided by the surrounding milieu could subsequently have allowed some degree of cuspal cytodifferentiation starting on day 4 in ovo. Similar compensatory mechanisms may have operated in Shh-null mice. Ongoing studies should provide further insights into these interesting issues.

EXPERIMENTAL PROCEDURES

Mouse Tooth Germ Transplantation

Mandibular fragments approximately 1 to 2 mm^2 and containing molar tooth germs were microdissected from E12–E14 CD-1 mouse embryos under sterile conditions and placed in serum-containing DMEM until transplantation. In some experiments, mesial cusp regions approximately 1 mm^2 in size were microdissected from E17 mouse embryo middle bell stage tooth germs. Fragments were transplanted to the wing buds of day 4 to 5 White Leghorn chick embryos in ovo. A portion of the dorsal ectoderm similar in size to the mouse tissue fragments was removed with a tungsten needle before transplantation with the aid of a dissecting microscope. Transplanted fragments were held in place with a small sterilized tungsten wire, which had been bent and firmly linked each fragment to host tissue, allowing close contact between donor and host mesenchymal tissues. After transplantation, eggs were sealed with transparent adhesive tape and reincubated.

Histology, Histochemistry, and In Situ Hybridization

At indicated times, chick embryos were killed and 4% buffered paraformaldehyde was injected into the limb with a syringe. Immediately thereafter, transplanted tissues were removed by microdissection and processed for histology and in situ hybridization as described (Noji et al., 1990). Briefly, paraffin-embedded serial tissue sections were pretreated with 1 μg/ml proteinase K (Sigma, St. Louis, MO) in 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid, pH 7.5, for 1 min at room temperature, immediately post-fixed in 4% paraformaldehyde buffer for 10 min, and then washed twice in 1$\times$ phosphate buffered saline (PBS) containing 2 mg/ml glycine for 10 min/wash. Sections were treated for 15 min with a freshly prepared solution of 0.25% acetic anhydride in triethanolamine buffer. Sections were hybridized with antisense or sense ^{35}S-labeled probes (approximately 1×10^6 DPM/section) at 50°C for 16 hr. After hybridization, slides were washed three times with 2$\times$ standard saline citrate (SSC) containing 50% formamide at 50°C for 20 min/wash, treated with 20 μg/ml RNase A for 30 min at 37°C, and washed three times with 0.1$\times$ SSC at 50°C for 10 min/wash. Sections were dehydrated by immersion in 70, 90, and 100% ethanol for 5 min/step, coated with Kodak NTB-3 emulsion diluted 1:1 with water, and exposed for 7 to 10 days. Slides were developed with Kodak D-19 at 20°C for 3 min, stained with hematoxylin and eosin, and analyzed and photographed with a Nikon microscope equipped for bright and dark field optics. A mouse DSPP cDNA clone encoding nucleotides 1243-1990 from the translated portion of the protein, and a full-length mouse amelogenin cDNA clone were prepared by reverse transcriptase-polymerase chain reaction (PCR) by using E18 mouse tooth germ total RNA as a template. PCR products were subcloned into the pBS SK(+) vector, and cDNA clone identity and sequence accuracy were based on previously published sequences

(MacDougall et al., 1997; Snead et al., 1985).

CELL PROLIFERATION ANALYSIS

At different times after implantation of mouse tooth germs, host chick embryos in ovo were injected with 10 μg/kg BrdU contained in 50 μl of PBS. Injection was performed subcutaneously in the trunk area of the embryos, making sure that no internal organ was damaged. Eggs were reincubated for 2 hr and were then fixed in 75% ethanol. Limbs were processed for immunohistochemical detection of incorporated BrdU by using a commercial kit (Amersham Pharmacia Biotec).

Antibody Treatment

Middle bell stage tooth germs isolated from E17 mouse embryos were further microdissected into fragments containing molar mesial cusps. Each fragment was microinjected with 1 μl of PBS containing 0.5 or 0.1 μg of Shh affinity-purified antibodies (Marti et al., 1995) in the vicinity of the cusps. Companion fragments were injected with 1 μl of PBS containing similar amounts of preimmune rabbit IgGs (Sigma). Each fragment was then implanted into day 4–5 chick embryo wing buds as above. Antibody microinjections were repeated 24 and 48 hr later. On day 3 and 4 from implantation, chick embryos were killed and limbs were fixed with 4% paraformaldehyde and processed for in situ hybridization. The tooth germ stage used in these experiments was selected to reduce the overall length of treatment and use the limited amounts of neutralizing antibodies available most effectively.

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