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Waking-up the Sleeping Beauty: Recovery of the Ancestral Bird Odontogenic Program

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ABSTRACT Recent advances in molecular and developmental genetics have provided tools for understanding evolutionary changes in the nature of the epithelial–mesenchymal interactions regulating the patterned outgrowth of the tooth primordia. Tissue recombination experiments in mice have identified the oral epithelium as providing the instructive information for the initiation of tooth development. Teeth were lost in birds for more than 80 million years ago, but despite their disappearance, a number of gene products and the requisite tissue interactions needed for tooth formation are found in the avian oral region. It is believed that the avian ectomesenchyme has lost the odontogenic capacity, whilst the oral epithelium retains the molecular signaling required to induce odontogenesis. In order to investigate the odontogenic capacity of the neural crest-derived mesenchyme and its potential activation of the avian oral epithelium, we have realized mouse neural tube transplantations to chick embryos to replace the neural crest cells of chick with those of mouse. Teeth are formed in the mouse/chick chimeras, indicating that timing is critical for the acquisition of the odontogenic potential by the epithelium and, furthermore, suggesting that odontogenesis is initially directed by species-specific mesenchymal signals interplaying with common epithelial signals. *J. Exp. Zool. (Mol. Dev. Evol.) 306B:227–233, 2006.*

Tooth development, as is the case during formation of many vertebrate organs, involves inductive and permissive interactions mediated by diffusible factors between the oral epithelium and the cranial ectomesenchyme (Kollar, '86). This mesenchyme derives from neural crest cells located at the caudal midbrain and rostral hindbrain (Trainor and Tam, '95; Imai et al., '96). During the initiation period, presumptive dental epithelium and mesenchyme become specified and the pattern of odontogenic loci established. The initiation period for tooth development in the mouse embryo starts at the embryonic day 8 (E8), when neural crest cells first emerge from the cranial neural folds. At E11, local thickenings of the oral epithelium form the dental placodes. The epithelium of the placodes then invaginates into the underlying ectomesenchyme to form the tooth bud (E12.5–13), around which, the mesenchyme proliferates and condenses, forming the dental papilla. At E14, the dental epithelium acquires the cap configuration, and by E16, the tooth germ has progressed to the bell stage. At this time, the tooth morphology is established and the epithelial and mesenchymal cells differentiate into enamel-secreting ameloblasts and dentin-producing odontoblasts, respectively.

Tissue recombination experiments between oral epithelial and ectomesenchymal tissues have identified the oral epithelium as providing the instructive information for the initiation of mouse tooth formation (Mina and Kollar, '87; Lumsden, '88). The E9–E11 presumptive dental epithelium can elicit tooth formation in neural crest-derived mesenchyme that does not normally participate in tooth formation. However, the presumptive dental epithelium is not able to induce odontogenesis in a mesenchyme that is not originated from neural crest, such as the limb mesenchyme (Mina and Kollar, '87; Lumsden, '88). The oral epithelium loses the odontogenic potential by E12: the

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ectomesenchyme can now instruct any kind of epithelium to form tooth-specific structures (Mina and Kollar, '87). These classical recombination experiments have indicated that the signals originated from oral epithelium are important for the initiation of mouse tooth formation. However, some controversy exists regarding the epithelium as the initial source of signals since heterospecific tissue recombination experiments have attributed the leading role to the mesenchyme (Lemus, '95).

Findings over the past few years have permitted the establishment of a model for the signaling pathways regulating inductive tissue interactions during murine tooth initiation (Thesleff and Sharpe, '97; Peters and Balling, '99). *Pitx2* is a homeobox gene that is initially expressed throughout the oral epithelium and progressively becomes restricted to the dental epithelium (Mucchielli et al., '97). Mice carrying a null mutation in this gene display tooth agenesis (Lin et al., '99). *Pitx2* is the earliest marker of the oral epithelium (E8.5) (Mucchielli et al., '97), while bone morphogenetic protein-4 (BMP4), fibroblast growth factor-8 (FGF8) and sonic hedgehog (Shh) are expressed later (between E9 and E11) and are involved in the determination of tooth-forming sites and the stepwise determination of ectomesenchyme into dental papilla (Vainio et al., '93; Hardcastle et al., '98; Tucker et al., '98, '99; Sarkar et al., 2000; Cobourne et al., 2004). BMP4 and FGF8 are responsible for the activation of the homeodomain-containing transcription factors Msx1 and Pax9, respectively, in the mesenchyme at the prospective sites of odontogenesis (Vainio et al., '93; Neubüser et al., '97; Tucker et al., '98). These transcription factors play a crucial role in the initiation phase of odontogenesis, since tooth development is arrested at the bud stage in mice deficient for either *Msx1* or *Pax9* (Satokata and Maas, '94; Peters et al., '98).

The Jurassic ancestral bird *Archaeopteryx* and certain birds of the Cretaceous possessed teeth with a typical conical morphology (Hou et al., '96), but none show details of their histology and tissue composition. Birds lost their dentition almost 80 million years ago, but a number of genes that initiate odontogenesis continue to be expressed in their maxillary and mandibular processes (Francis-West et al., '98; Schneider et al., '99). Rudimentary local epithelial ingrowths, which share similarities in organization and morphology with tooth primordia, are formed transiently in the mandibular and maxillary arches of the avian embryos (Romanoff, '60; Chen et al., 2000). Although these epithelial thickenings closely resemble the mouse dental thickenings, the molecular mechanisms regulating their outgrowth appear to be different since their development is arrested at this stage. This may be due to differences in neural crest cells and/or in oral epithelium. It has been shown that a number of genes, which remain silent in birds and are participating in tooth formation, can be reactivated upon appropriate signaling (Wang et al., '98). In vitro recombination experiments have shown that chick epithelium cultured with mouse dental mesenchyme produced dental structures. These results suggest that the cranial neural crest cells of birds have lost odontogenic capacity, whereas the oral epithelium retains the signaling properties to induce odontogenesis in a competent mesenchyme (Kollar and Mina, '91; Wang et al., '98). Our previous findings demonstrated that chimeric teeth are developing in ovo after mouse neural crest transplantation in chick embryos (Mitsiadis et al., 2003). The aim here is to identify unequivocally that the interacting cells forming the chimeric teeth are respectively mouse and chick. In particular, we aim to test the stage-specific competence of neural crest-derived mesenchyme in relation to gene expression in the epithelium.

MATERIALS AND METHODS

Mouse/chick chimeras

JA657 chick embryos at 1 day of incubation (7 somites), and Swiss mice embryos at E8 (4–6 somites) or E9 (20 somites) were used. Reciprocal exchanges of precisely defined regions of the neural tube were performed between chick and mouse embryos as previously described (Mitsiadis et al., 2003). The cephalic region of the neural tube was removed from the chick host and replaced by the mouse donor graft. Chimeric embryos were incubated in ovo for different time periods.

Distinction between mouse- and chick-derived tissues in chimeras

Heads of chimeras were prepared for histological examination and in situ hybridization. To ascertain the presence of donor tissue in hosts, several sections were analyzed after Feulgen-Rossenbeck or Hoechst staining to distinguish between DNA repartition in mouse and chick nuclei (mouse cells have a more intense nuclear staining). Most of the

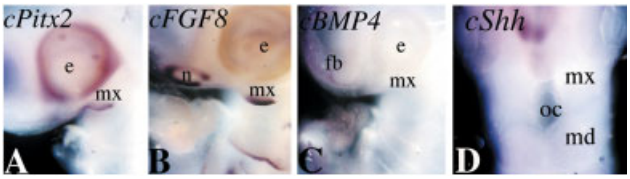

Fig. 1. Detection of genes expressed in the chick oral epithelium. Whole mount in situ hybridization. (**A** and **B**) Lateral view of stage-21 chick embryos showing chick *Pitx2* (*cPitx2*) expression throughout the oral epithelium (A), and a much more restricted expression of the chick *FGF8* gene (*cFGF8*; B). (**C** and **D**) Lateral (C) and frontal (D) views of stage-21 chick embryos showing the absence of chick *BMP4* (*cBMP4*; C) and chick *Shh* (*cShh*; D) in oral epithelium. Note the presence of *cBMP4* transcripts in the forebrain (C). Abbreviations: e, eye; fb, forebrain; md, mandibular process; mx, maxillary process; n, nose; oc, oral cavity.

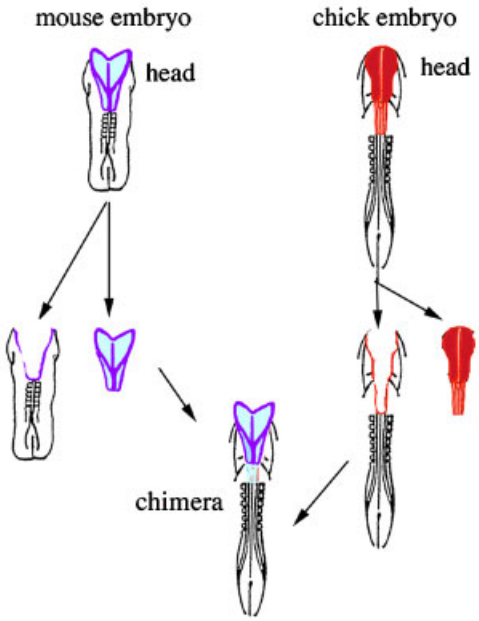

Fig. 2. Experimental procedure of mouse neural tube transplantation into chick host embryos. The 6-somite-stage chick encephalon was replaced by an equivalent part of the E8 (4–6 somites) mouse encephalon (homochronic graft).

sections with tooth-like forming structures were hybridized with mouse-specific fluorescein-labeled *Msx1* and *Pax9* probes that recognize mouse cranial neural crest populations and chick-specific *FGF8*, *Shh*, *BMP4* and *Pitx2* digoxigenin-labeled probes that recognize chick oral epithelial structures.

RESULTS AND DISCUSSION

We have examined the expression in the chick embryonic jaw of molecules known to be involved in the initiation of murine tooth formation. We found that while chick *Pitx2* and *FGF8* were expressed in the oral epithelium of the Hamburger and Hamilton stage 21 chick embryo (corresponding to an E10.5 mouse embryo) (Fig. 1A and B), the chick *BMP4* and *Shh* genes were not expressed (Fig. 1C and D). These results suggest that the information for tooth initiation is partly present in the chick mandibular arch at this stage. It has been shown that ectopic BMP4 expression in oral epithelium of stage 23–25 chick embryos activates tooth-specific genes in the mandibular mesenchyme and leads to the formation of tooth-like structures (Chen et al., 2000).

The findings described above lead one to pose several key questions: Does mesenchyme-derived signaling play a critical role in the initiation of tooth formation? Does chick oral epithelium have the capacity to form tooth structures in ovo when provided with appropriate mesenchymal signals?

It has been shown previously that cranial neural crest cells start migrating at E8 (Nichols, '81), well before *Pitx2*, *FGF8* and *BMP4* expression (E9–E10) in the oral epithelium (Mucchielli et al., '97; Tucker et al., '98, '99). These neural crest cells are apparently pluripotent and according to previous findings they acquire specific signals from epithelium to stimulate their odontogenic potential. Their migration into the mandibular and maxillary processes continues through the 11 somite stage (E8.5) and is already complete by E9 (Lumsden and Buchanan, '86). Since this is the earliest stage from which tissue has been used for recombination experiments (Mina and Kollar, '87; Lumsden, '88), it is likely that the E9 oral epithelium had already acquired a pre-pattern as a consequence of a prior interaction with neural crest cells. Taken together, these results suggest that neural crest cells may play an equal primary role in initiation of the odontogenic program whereby they induce and/or maintain oral epithelial expression of *Pitx2*, *BMP4*, *FGF8* and *Shh*.

Interspecific homotopic neural tube transplantations were performed to investigate the odontogenic capacity of mouse ectomesenchymal cells

(Mitsiadis et al., 2003). The rostral murine neural tube was transplanted into a chick host in which the equivalent tissues had been ablated (Fig. 2). This transplantation has been realized prior to the closure of the neural tube because it contains all the pre-migratory cranial neural crest cells. The mouse/chick chimeras showed ingrowths of the oral epithelium with bud and cap configurations (Fig. 3A and B). Mineralized structures resembling tooth germs are observed beneath the oral epithelium at more advanced developmental stages (14 days post-surgery) (Fig. 3C). The deposition of the mineral matrix is similar to the dentin deposition observed in developing mouse teeth: deposition starts at the tip of the cusp and proceeds apically. In the anterior part of the oral epithelium, we also detected many ingrowths into the mouse ectomesenchyme with unusual shapes.

Mouse neural crest cells invade the maxillary and mandibular processes of the chick host by 1–2 days post-surgery (Fig. 4A and B) (Mitsiadis et al., 2003). These cells contribute to the formation of

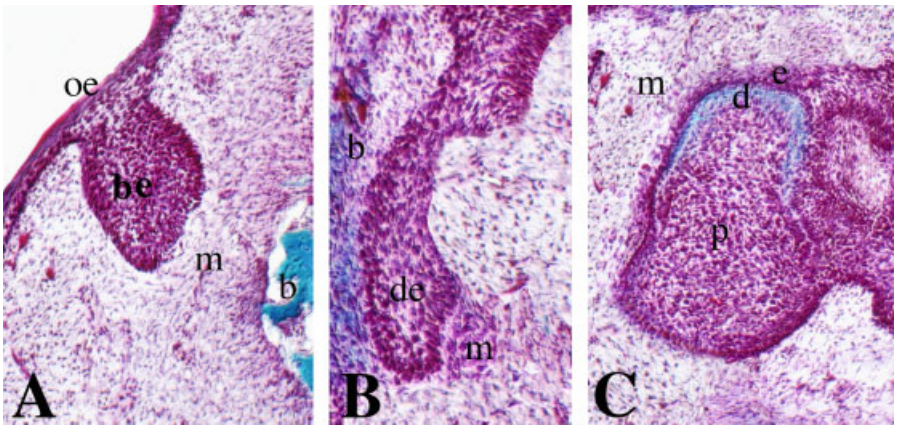

Fig. 3. Formation of mineralized dental-like structures in a mouse/chick chimera, 7 (A), 9 (B) and 14 (C) days post-surgery. (**A**, **B**) Histological section of oral epithelial invaginations having a bud (A) and a cap (B) configuration. (**C**) A mineralized structure resembling a tooth germ. The mineralized matrix is evident after Masson's trichrome staining. Note the formation of a single cusp (conical shape) and the absence of enamel matrix and polarized epithelial cells. Abbreviations: b, bone; be, bud epithelium; d, dentin; de, dental epithelium at the cap stage; e, dental epithelium; oe, oral epithelium; m, jaw mesenchyme; p, dental papilla mesenchyme.

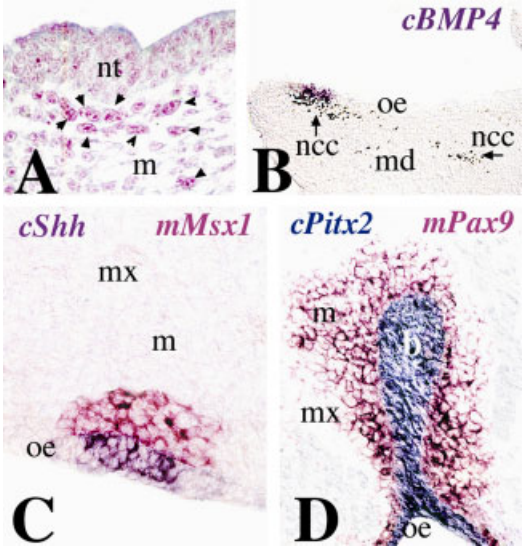

Fig. 4. Localization of mouse neural crest cells in the forming maxillary and mandibular processes of mouse/chick chimeras. Mouse cells detected after Feulgen-Rossenbeck (A) or Hoechst (B) staining in transverse sections of the head region of mouse/chick chimeras, 1–2 days post-surgery. Examination of the chick origin and dental potential of the epithelial ingrowths by in situ hybridization using either fluorescein- (C, D) (red color) or digoxigenin-labeled (B–D) (violet color) species-specific probes. (**A**) A population of mouse neural crest cells (arrowheads) leaving the grafted neural tube are detected after Feulgen-Rossenbeck staining. (**B**) Migration and localization of mouse neural crest cells (black cells, arrows) in the mandibular process 2 days post-grafting after Hoechst staining and computer imaging color alteration. Chick cells of the forming dental placode, which overly the mouse neural crest cells, express the chick *BMP4* (*cBMP4*) gene. (**C**) Expression of chick *Shh* (*cShh*) in a restricted area of the oral epithelium, overlying mouse *Msx1* (*mMsx1*) expressing mesenchymal cells, 3 days post-surgery. (**D**) Expression of chick *Pitx2* (*cPitx2*) in the bud epithelium, 9 days post-grafting. Mouse *Pax9* (*mPax9*) expression in the mesenchyme surrounding the epithelial bud. Abbreviations: b, bud; m, mesenchyme; md, mandibular process; mx, maxillary process; ncc, neural crest cells; nt, neural tube; oe, oral epithelium.

tooth-like germ structures at different time points after grafting. The heparin-binding growth/differentiation factor Midkine (MK) is expressed in mouse neural crest cells during embryogenesis (Mitsiadis et al., '95a) and subsequently expression becomes restricted in dental tissues (Mitsiadis et al., '95b). In the chimeras, *MK* is expressed in mouse neural crest cells migrating into the maxillary and mandibular processes of the chick host, while the mouse *Pax9* and *Msx1* genes are expressed only in those cells contacting the chick oral epithelium (Mitsiadis et al., 2003). Expression of *Pax9* and *Msx1* is limited to mouse dental mesenchyme during odontogenesis (Vainio et al., '93; Neubüser et al., '97; Tucker et al., '98), suggesting that neural crest cells expressing these genes possess odontogenic potential. The reciprocal interactions between mouse neural crest-derived mesenchyme and chick oral epithelium are thus responsible for the development of tooth structures in the chimeras, indicating that the loss of teeth in birds is probably due to the lack of appropriate signaling molecules from the neural crest.

Mouse cranial neural crest cells contain signals that can induce *BMP4* and *Shh* expression in the chick oral epithelium, not normally expressed here. In situ hybridization in chimeras, 2 days after the neural tube transplantation, showed localized regions of epithelial chick *Shh* and *BMP4* expression that correspond to the sites overlying mouse neural crest cells expressing the mouse *MK*, *Msx1* and *Pax9* genes (Fig. 4B and C). These results indicate that neural crest cells may have a significant role in tooth initiation through the activation of *BMP4* and *Shh* expression in oral epithelium. When the chick oral epithelium acquires the bud configuration in the chimeric embryos (7 days post-surgery), ectomesenchymal cells surrounding the epithelial ingrowths express the tooth-specific genes *Msx1* and *Pax9* of the mouse (Fig. 4D). The detection of the chick *Pitx2* gene indicates the origin of the bud epithelium (Fig. 4D). At more advanced developmental stages showing clear morphological evidence of tooth formation, chick *Pitx2* and mouse *Msx1* are expressed in the epithelium and mesenchyme, respectively, of the chimeric tooth germs (Mitsiadis et al., 2003). The dentin-specific non-collagenous extracellular matrix protein DSP (dentin sialoprotein) (Butler et al., '92) has also been detected in the chimeric teeth (Mitsiadis et al., 2003). *Barx1* is not expressed in the mesenchyme of the chimeric teeth (data not shown). In mice, *Barx1* expression is restricted to the mesenchyme of developing molars, and is never seen in the mesenchyme of the incisors (Mucchielli et al., '97). Thus the eventual loss of *Barx1* expression in the chimeric teeth provides evidence of restricted morphogenetic information leading to coniform tooth structures, resembling reptilian or ancestral avian teeth (Stock, 2001; Herrel et al., 2004).

Enamel matrix is not detected in the chimeric tooth germs, and the amelogenin protein, which is thought to be involved in the regulation of enamel crystallite formation, was absent from epithelial cells overlying the dentin matrix. The origin and nature of the epithelial-derived matrix, which was covering the dentin matrix in ancient birds, remains controversial and unresolved. Fossils cannot provide a reliable source of information regarding the composition of the crown in ancient birds. Interestingly enough, the homologs of the mammalian enamelin and amelogenin genes have not been detected in the chick genome (Kawasaki et al., 2004), while amelogenin is present in reptiles and amphibians (Toyosawa et al., '98; Sire et al., 2006). However, Chiappe and Chinsamy ('96) have suggested that teeth of the Early Cretaceous *Pterodaustro* contained non-prismatic enamel, as observed in extant reptiles (Sander, 2001), but the evidence for enamel formation was based on morphological rather than molecular criteria. Previous in vitro recombinations of E16–E18 mouse dental mesenchyme and chick oral epithelium have shown that the chick tissue responded by forming enamel (Kollar and Fisher, '80), but the interpretation of these heterospecific recombinations remains uncertain because of the possible contamination of the mouse mesenchyme with mouse odontogenic epithelial cells: it is the latter cells that would produce mouse-type enamel in these heterospecific explants.

We finally tested whether mouse neural crest cell transplantations taken from different axial levels have the ability to induce tooth formation in mouse/chick chimeras. Previous tissue recombination studies have shown that any kind of neural crest-derived mesenchyme can participate in tooth formation (Lumsden, '88), indicating that neural crest cells display plasticity to respond to changing environmental signals. It is now clearly established that the degree of neural crest plasticity is dependent upon the developmental age and size of the transplant (Trainor and Krumlauf, 2001). While E9 caudally derived mouse neural crest cells will migrate into the facial region, ingrowths of the oral epithelium and tooth-specific molecular

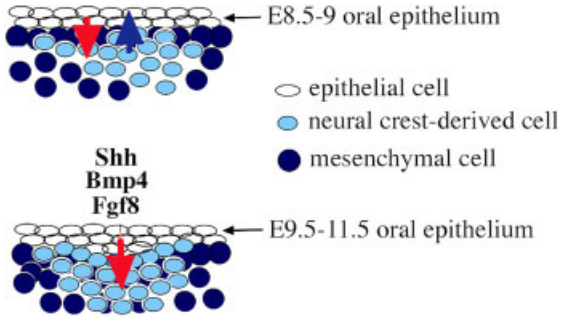

Fig. 5. Schematic illustration of a hypothetical model on the initiation of mouse odontogenesis. Neural crest cells may play a primary role in initiation of the odontogenic program by inducing and/or maintaining oral epithelial expression of *BMP4*, *FGF8* and *Shh*. Cephalic neural crest cells, which are migrating into the mandibular and maxillary processes, are initially interacting with the oral epithelium between E8.5 and E9. This time window is very important for the exchange of yet unknown signaling molecules between these two tissues and the acquisition of the odontogenic potential by the epithelium. Thereafter, the pre-patterned oral epithelium (E9.5–E11.5) can instruct any neural-crest-derived mesenchyme to form teeth.

markers are not detected in these chimeras (data not shown). These findings suggest that at this stage in development only cranial neural crest cells possess the potential to contribute to the formation of tooth-specific structures and that caudally derived mouse neural crest cells are more likely to be already irreversibly committed to their caudal identity.

Taken together, these results suggest that the differences in facial development between avian and mammalian embryos rely on species-specific neural crest cell-derived signals. The in ovo experimental approach used here, combined with molecular tools, clarifies previous findings and suggests that cranial neural crest cells contain the odontogenic potential and contribute equally with the oral epithelium to the initiation of tooth formation (Fig. 5). Moreover, the data present a striking example of the retention of an evolutionary dormant developmental genetics program, specifically in the oral epithelium, which can still be reactivated.

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