Development of teeth in chick embryos after mouse neural crest transplantations

Thimios A. Mitsiadis,*† Yvonnick Chéraud,§ Paul Sharpe*, and Josiane Fontaine-Pérus§

*Department of Craniofacial Development, Guy’s, King’s, and St. Thomas’ Dental Institute, King’s College London, Floor 28 Guy’s Tower, Guy’s Hospital, London SE1 9RT, United Kingdom; †Centre National de la Recherche Scientifique Unité Mixte de Recherche 6018, Faculté des Sciences et des Techniques, Université de Nantes, 2 Rue de la Houssinière, BP 92208, 44322 Nantes Cedex 3, France; and §Laboratoire de Biologie Moléculaire et Cellulaire, Unité Mixte de Recherche 5665, Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, 46 Allée d’Italie, 69364 Lyon Cedex 07, France

Edited by N. M. Le Douarin, Institut d’Embryologie Cellulaire et Moléculaire du Centre National de la Recherche Scientifique, Nogent-sur-Marne Cedex, 46 Allée d’Italie, 69364 Lyon Cedex 07, France

Teeth were lost in birds 70–80 million years ago. Current thinking holds that it is the avian cranial neural crest-derived mesenchyme that has lost odontogenic capacity, whereas the oral epithelium retains the signaling properties required to induce odontogenesis. To investigate the odontogenic capacity of ectomesenchyme, we have used neural tube transplantations from mice to chick embryos to replace the chick neural crest cell populations with mouse neural crest cells. The mouse/chick chimeras obtained show evidence of tooth formation showing that avian oral epithelium is able to induce a nonavian developmental program in mouse neural crest-derived mesenchymal cells.

Tooth development, in common with the formation of many other vertebrate organs, involves reciprocal series of epithelial–mesenchymal interactions (1). These inductive interactions are mediated by diffusible factors between oral epithelium and neural crest-derived mesenchyme (2, 3). In the mouse embryo, the initiation period of tooth development extends from embryonic day (E) 8, when crest cells first emerge from the cranial neural folds, to E11, when local thickenings of the oral epithelium are formed (4–6). The epithelium then invaginates into the underlying mesenchyme to form the tooth bud (E12.5–13).

Classical tissue recombination experiments and more recent molecular analysis have identified the oral epithelium as providing the instructive information for the initiation of mouse tooth development. The E9–11 presumptive dental epithelium can elicit tooth formation even in neural crest-derived mesenchyme that does not normally form teeth, but is not able to induce tooth formation in a nonneural crest-derived mesenchyme, such as the limb mesenchyme (4, 7). By E12, the odontogenic potential shifts to the mesenchyme, which can instruct any kind of epithelium to form tooth-specific structures (7–9). These experiments point to the importance of epithelial signals in the initiation of mouse tooth formation.

Birds lost their dentition almost 70–80 million years ago, but a number of genes that initiate odontogenesis continue to be expressed in their jaws (10, 11). During avian embryonic development, rudimentary structures consisting of local epithelial thickenings are formed transiently in the mandibular arch (12, 13), and these thickenings closely resemble the murine dental thickenings. Although these epithelial ingrowths share similarities in organization and morphology with mouse tooth primordia, the molecular mechanisms regulating their outgrowth appear to be distinct because their development is arrested at this stage, which may be due to the origin of the neural crest cells and/or regional differences in the oral epithelium. Several in vitro studies have suggested that a number of genes involved in tooth formation, and which remain silent in modern birds, can be reactivated upon appropriate signaling (8, 14). Isolated chick epithelium cultured in combination with mouse tooth mesenchyme produced dental structures, suggesting that it is the cranial neural crest-derived mesenchyme of modern Aves that has lost odontogenic capacity, whereas the oral epithelium retains the signaling properties to induce odontogenesis in competent mesenchyme (8, 14, 15). Unfortunately, such recombination approaches suffer from being performed in vitro, and moreover, it is difficult to eliminate completely contamination of mouse mesenchyme with residual epithelium. To determine whether teeth can form in the developing jaws of avian embryos in ovo, we grafted mouse cranial neural crest in place of chick.

Materials and Methods

Preparation of Mouse/Chick Chimeras. JA657 chick embryos at 1 day of incubation (seven somites) and Swiss mice embryos at E8 (four to six somites) were used for the microsurgery. Reciprocal exchanges of precisely defined regions of the neural tube were performed between chick and mouse embryos as previously described (16). The cephalic region to be grafted was first delimited in the mouse donor and the chick host, then removed from the host and replaced by the donor graft (Fig. 1 Upper). Chimera embryos were incubated in ovo for different periods (1–18 days).

Visualization of Neural Crest Cell Migration. Chimeras killed 1–2 days after surgery were prepared for whole-mount in situ hybridization with mouse probes to check mouse neural crest cell migration into the facial territories of the chick host embryos. Heads of older chimeras were prepared for histological examination and in situ hybridization on sections. To ascertain the presence of donor tissue in hosts, several sections of each chimera were stained with bis-benzimidze (Hoechst staining), as described (16). The distribution of mouse neural crest cells in the head of chimeras was visualized by a DNA repartition that differs in the mouse and chick nuclei. (Mouse cells had a brighter appearance after Hoechst staining.)

In Situ Hybridization and Immunohistochemistry on Tissue Sections. For in situ hybridization, digoxigenin-labeled mouse and chick specific riboprobes (mMK, mMx1, mPax9, mBarx1, cPitx2, cFGF8, cBMP4, cShh) were used. Whole-mount in situ hybridization and in situ hybridization on cryosections were performed according to Wilkinson (17).

For immunohistochemistry, affinity-purified antibodies against several proteins (dentin sialoprotein, nestin) were used. Immunoperoxidase (ABC Kit, Vector Laboratories) staining was performed as previously described (18, 19). Positive peroxidase staining produces red color on light microscopy.

Results and Discussion

Several molecules are well known to be involved in the initiation of murine tooth formation. Pitx2 is a homeobox gene that is...
initially expressed throughout the mouse oral epithelium and progressively becomes restricted to the dental epithelium (20). Bone morphogenetic protein-4 (BMP4), fibroblast growth factor-8 (FGF8), and sonic hedgehog (Shh) are involved in the determination of tooth-forming sites in mice and the stepwise determination of ectomesenchyme into dental papilla (21–25). Expression of BMP4 and FGF8 has been documented in chick oral epithelium during chick development, it is not expressed in early (st21) dental epithelium (11, 26). The restricted expression of these molecules to dental placodes in oral epithelium during mouse development is thus an indication of the induction of odontogenesis.

To investigate the odontogenic capacity of murine ectomesenchymal cells and also to examine whether avian oral epithelium can undergo full tooth development, we performed a series of interspecific homotopic neural-tube transplantations. The whole dorsoventral aspect of the rostral murine neural tube, before its closure (when it still contains all of the premigratory cranial neural crest cells), was transplanted into an avian host from which the equivalent tissues had been surgically removed (Fig. 1 Upper). Mouse neural crest cells had already invaded the maxillary and mandibular processes of the chick host by 1 to 2 days after surgery (Fig. 2A). These cells contribute to the formation of tooth-like germ structures at different time points after grafting in the chick hosts (Fig. 2B–D and Fig. 1 Lower). Transplants of prosencephalon-mesencephalon (series B) and prosencephalon-mesencephalon–rhombencephalon (series C) both contributed to the formation of tooth structures in the mouse/chick chimeras, whereas no tooth structures were obtained from transplants of prosencephalon (series A).

We undertook a molecular analysis to examine expression of both early and late markers of tooth development in chimeras. Midkine (MK) is a heparin-binding growth/differentiation factor that is expressed in all neural crest cells during the early stages of mouse embryonic development (18). Subsequently, MK expression becomes down-regulated in the vast majority of tissues and organs, but its expression persists in the developing murine teeth until E18 (late bell stage) (19). In chimeric embryos, MK was found to be expressed in a large population of mouse neural crest cells migrating into the maxillary and mandibular processes of the chick host (Fig. 3 A and C). Expression of the mouse homeodomain-containing transcription factors Msx1 and Pax9 was far more restricted, occurring specifically in those cell populations surrounding or contacting the chick oral epithelium (Fig. 3D–F). These findings suggest that neural crest cells expressing Pax9 and Msx1 with MK possess odontogenic potential, because the expression of Pax9 and Msx1 is limited to dental mesenchyme from the earliest stages of murine odontogenesis (21, 22, 25). We conclude that the reciprocal signaling interactions between neural crest-derived mesenchyme from mouse and localized regions of the chick oral epithelium are responsible for the subsequent elaboration of the specific form and structure of the teeth described above. The loss of teeth in Aves is thus probably due to the lack of appropriate neural crest-derived signaling molecules involved in epithelial–mesenchymal interactions.

It had been suggested that loss of BMP4 in chick “vestigial” tooth rudiments is responsible for their failure to progress into
teeth (13). To test if mouse cranial neural crest contains specific signals that can ectopically induce localized BMP4 and Shh expression in the chick oral epithelium, we performed in situ hybridization in chimeras, 2 to 4 days after surgery. Although Pitx2 was expressed throughout the oral epithelium (Fig. 4 F and L), ectopic chick FGF8, Shh, and BMP4 expression was restricted to those areas that form the epithelial placodes described above (Fig. 4 C–E, G, and I). These localized regions of epithelial expression correspond to the sites overlying mouse neural crest cells (Fig. 4 A, B, and K) that express the mouse Mxsl, Barx1, and MK genes (Fig. 4 H, J, and M). These results indicate that, in vivo, neural crest cells may play a role in the activation of BMP4 and Shh expression in tooth-forming sites of the murine oral epithelium.

Seven to nine days after grafting, the chick oral epithelium had invaginated into the underlying ectomesenchyme and acquired a bud configuration (Fig. 5 A and B). As during the bud stage of mouse tooth development, mouse ectomesenchymal cells surrounding the chick epithelial invaginations expressed the tooth-specific genes Mxsl, Pax9, and Barx1 (Fig. 5 C, D, and G). Although each of these genes is expressed in cells other than dental mesenchyme, dental mesenchyme cells are the only cells in the embryo that express all three genes. MK expression, which was initially widespread in the migrating neural crest cells, became restricted around the invaginated chimera epithelium (Fig. 5 F). The oral origin of the epithelium was evident from the detection of the chick Pitx2 gene (Fig. 5 E). The differential growth and subsequent folding of the dental epithelium is thought to be directed by a transient signaling center known as the enamel knot (2, 3, 26). Shh expression has been reported in the enamel knot of developing mouse teeth (27). Similarly, ectopic chick Shh expression was seen in a restricted population

Fig. 4. Formation of dental placodes in the oral epithelium of mouse/chick chimeras. Hoechst staining (A, B, and K) and digoxigenin in situ hybridization on sections of 2 (A–F) to 4 (G–M) days after grafting chimeras, by using chick epithelial (C–G, I, and L) and mouse mesenchymal (H, J, and M) markers. (A and B) Mouse cells appear brighter after Hoechst staining in the maxillary and mandibular processes. (C–E) Expression of chick FGF8 (cFGF8; C), Shh (cShh; D), and BMP4 (cBMP4; E) in restricted areas of the oral epithelium. (F) Expression of chick Pitx2 (cPitx2) in all cells of the oral epithelium. Compare C, D, E, and F with A and B, showing the arrival of mouse neural crest cells at the sites of cFGF8, cShh, and cBMP4 expression. (G)Restricted cShh expression in oral epithelium. The asterisk shows the mesenchymal area in which the mouse Barx1 gene (mBarx1) was detected in an adjacent section. (H) Expression of the mBarx1 gene in mesenchymal cells that are in contact with cShh-expressing epithelial cells (asterisk). (I and J) Similarly, the chick epithelium expressing cFGF8 (I, asterisk in J) is in contact with the mesenchyme in which the mMK gene was expressed (J, asterisk in I). (L) Expression of cPitx2 to the oral epithelium of maxillary and mandibular processes. Note the absence of the hybridization signal in the aboral epithelium. (M) Mouse Mxsl (mMxsl) expression in a population of mesenchymal cells in contact with the oral epithelium of the maxilla, ae, aboral epithelium; md, mandibular process; mx, maxillary process; oe, oral epithelium.
expression of the chick in mesenchymal cells surrounding the epithelial bud structures. Restricted to tooth bud; tc, tooth cap-like invagination. (C, D, F, and G) Detection of mouse Msx1 (mMsx1; C), Pax9 (mPax9; D), MK (mMK; F), and Barx1 (mBarx1; G) transcripts in mesenchymal cells surrounding the epithelial bud structures. Restricted expression of the chick Shh gene (cShh) to cells of the epithelial bud structure (G; brown color, arrow) that represents the equivalent population of cells forming the "enamel knot" in developing mouse teeth. (E) Expression of the chick Pitx2 gene (cPitx2) in the bud epithelium. ek, enamel knot; oe, epithelium; m, mesenchyme; md, mandibular process; mx, maxillary process; tb, tooth bud; tc, tooth cap-like invagination.

Fig. 5. Formation of dental-like epithelial bud structures in mouse/chick chimeras. The chick oral epithelium invaginates into the underlying mouse neural crest-derived mesenchyme and acquires bud configurations, 7–9 days after grafting. Hoechst staining (A and B) and in situ hybridization on sections by using tooth-specific molecular markers (C–G). Examination of the chick origin and dental potential of the epithelial ingrowths by in situ hybridization by using either fluorescein-labeled (G, brown color) or digoxigenin-labeled (C–G, violet and blue colors) species-specific probes. (A and B) Visualization of mouse neural crest cells surrounding chick epithelial bud/cap structures (arrows) in the maxillary processes. (C, D, F, and G) Detection of mouse Msx1 (mMsx1; C), Pax9 (mPax9; D), MK (mMK; F), and Barx1 (mBarx1; G) transcripts in mesenchymal cells surrounding the epithelial bud structures. Restricted expression of the chick Shh gene (cShh) to cells of the epithelial bud structure (G; brown color, arrow) that represents the equivalent population of cells forming the "enamel knot" in developing mouse teeth. (E) Expression of the chick Pitx2 gene (cPitx2) in the bud epithelium. ek, enamel knot; oe, epithelium; m, mesenchyme; md, mandibular process; mx, maxillary process; tb, tooth bud; tc, tooth cap-like invagination.

of epithelial cells in chimera tooth germs (Fig. 5G), indicating the presence of a tooth-shape regulator in these structures.

Fourteen days after grafting, multiple ingrowths of the oral epithelium were evident, and mineralized structures resembling tooth germs were observed beneath the oral epithelium (Fig. 6C). Chick oral epithelium invaginates into the underlying mouse neural crest-derived mesenchyme and acquires bud configurations, 7–9 days after grafting. Hoechst staining (A and B) and in situ hybridization on sections by using tooth-specific molecular markers (C–G). Examination of the chick origin and dental potential of the epithelial ingrowths by in situ hybridization by using either fluorescein-labeled (G, brown color) or digoxigenin-labeled (C–G, violet and blue colors) species-specific probes. (A and B) Visualization of mouse neural crest cells surrounding chick epithelial bud/cap structures (arrows) in the maxillary processes. (C, D, F, and G) Detection of mouse Msx1 (mMsx1; C), Pax9 (mPax9; D), MK (mMK; F), and Barx1 (mBarx1; G) transcripts in mesenchymal cells surrounding the epithelial bud structures. Restricted expression of the chick Shh gene (cShh) to cells of the epithelial bud structure (G; brown color, arrow) that represents the equivalent population of cells forming the "enamel knot" in developing mouse teeth. (E) Expression of the chick Pitx2 gene (cPitx2) in the bud epithelium. ek, enamel knot; oe, epithelium; m, mesenchyme; md, mandibular process; mx, maxillary process; tb, tooth bud; tc, tooth cap-like invagination.

chimera tooth germs (Fig. 6C and E) and in several unusual structures formed at the anterior part of the oral epithelium (Fig. 6D). Chick Pitx2 was not expressed in the epithelial cells contacting the mineralized matrix, which is reminiscent of Pitx2.
down-regulation in ameloblasts observed in mouse teeth. The mouse Msx1 (Fig. 6F) and the chick Shh genes (Fig. 6G) were expressed, respectively, in dental mesenchyme and epithelium of the chimera teeth. Dentin sialoprotein, a noncollagenous extracellular matrix protein produced by odontoblasts (28) and osteoblasts (29), was detected in ectomesenchymal cells and the mineralized matrix (Fig. 6H). The intermediate filament protein nestin, a marker of neurons, muscles (30) and differentiating and mature odontoblasts (31), was also detected in cells producing dentin matrix in the chimera tooth germs (Fig. 6I and J). The presence of both these markers together in the same cell layer provides a strong indication that these cells are odontoblasts.

These results show that, although within a species cranial neural crest cells do not appear to be prepatterned with respect to their skeletal fates, they do contain the information to interpret generic epithelial signals and to behave in a species-specific way.

We thank W. Butler (University of Texas, Houston) for the dentin sialoprotein antibody; U. Lendahl (Karolinska Institute, Stockholm) for the nestin antibody; S. Artavanis-Tsakonas, N. Le Douarin, P. Lemaire, M. Maroto, and O. Pourquie for valuable comments; K. Dale for comments and improving the manuscript; and M. Zampieri for help with Masson's trichrome staining. This work was partly realized at the Institut de la Biologie du Développement de Marseille, in the laboratory of C. Goridis. The research was supported by grants from the Association pour la Recherche sur le Cancer, Association Française contre les Myopathies, Centre National de la Recherche Scientifique, the Wellcome Trust, Medical Research Council, and the University of Nantes.