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Potential Contribution of Neural Crest Cells to Dental Enamel Formation

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Abstract

Neural crest cells (NCCs) are a multipotent embryonic cell population that contributes to the formation of various craniofacial structures including teeth. It has been generally believed that dental enamel is an ectodermal derivative, whereas the dentin-pulp complex and the surrounding supporting tissues originate from NCC-derived mesenchyme. These traditional concepts stem mainly from several early studies of fishes and amphibians. Recently, *Wnt1-Cre/R26R* mice, a mouse model for NCC lineage analysis, revealed the contribution of NCCs to mammalian tooth development. However, the discrepancy of expression patterns between different NCC-specific transgenic mouse lines makes it compulsory to revisit the cell lineage in mammalian tooth development. Here, we reevaluated the NCC lineage during mouse tooth development by using *P0-Cre/R26R* mice, another NCC-specific transgenic mouse line. Inconsistent with the traditional concepts, we observed the potential contribution of NCCs to developing enamel organ and enamel formation. We also demonstrated that the *P0-Cre* transgene was specifically expressed in migrating NCC in the hindbrain region, where NCC contributes to tooth, validating their applicability for NCC lineage analysis. Our unanticipated finding may change the general understanding of tooth development and provide new insights into dental stem cell biology.

Keywords

ROSA26R reporter; Transgenic mice; Neural crest; Tooth development; Enamel organ

INTRODUCTION

Understanding of developmental biology, stem cell biology, and tissue engineering greatly influences the progression of regenerative medicine [1; 2]. Particularly, comprehension in origins of cells, tissues, and organs may lead to the discovery of clues to identify stem cells in specific adult organs and to propose feasible regenerative strategies [3; 4; 5]. For the past decades, significant efforts have been made to regenerate teeth for replacement of missing teeth, although only limited success has been achieved [6; 7; 8; 9]. With ultimate goal of regenerating functional teeth, analyzing the cell lineages of tooth development and

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unraveling the underlying mechanisms of this developmental process will not only gain insights into the general development principals of organogenesis but also shed light on regenerative dentistry. Tooth development is a sequential process of epithelial-mesenchymal interactions that involves intricate modulation of complex signaling pathways [10; 11]. During early embryogenesis, neural crest cells (NCCs), a multipotent cell population, migrate from the crests of the neural folds into the ventral embryo and give rise to various cell, tissue, and organ structures throughout the whole body, especially the major part of mesenchymal cell population in craniofacial region [12; 13; 14]. Particularly, early transplantation studies on amphibians demonstrated that cranial NCCs contribute to the developing dental mesenchyme [15; 16]. Also, DiI-labeling NCC tracing experiments with long-term culture of rat embryos revealed the NCC contribution to mammalian tooth development [17]. More recently, cell lineage analysis by using *Cre-loxP* DNA recombination system has been applied in the research of developmental biology. By crossing tissue-specific *Cre* mouse lines with reporter lines, the specific cell population of interest can be permanently marked and traced. To date, there are several NCC-specific *Cre* mouse lines for NCC lineage analyses. The *Wnt1-Cre* transgenic mice are widely used as a NCC-specific *Cre* mouse line [18; 19]. In combination with a *Cre* reporter line *R26R* [20], it is shown that NCCs contribute to the formation of dentin-pulp complex rather than dental enamel in tooth development [21], which is consistent with the classical observation from fish and amphibians. However, although all these transgenic lines are claimed to be NCC-specific, their expression patterns are not completely identical [19; 22; 23; 24]. Therefore, to get reliable and comprehensive results, comparative analyses by using two or more transgenic lines for NCC lineage tracing have been conducted in many research fields, such as heart development [4], neurobiology [25], and mesenchymal stem cell biology [25; 26]. In terms of NCC contribution to mammalian tooth development, only one piece of evidence has been shown by the NCC-specific *Wnt1-Cre* transgenic mice [18; 19; 21]. Accordingly, we consider it imperative to reevaluate NCC contribution to tooth development with another NCC-specific mouse line. Therefore, in this study, we used *P0-Cre* [24], another NCC-specific *Cre* mouse line, to reexamine the NCC lineage in mammalian tooth development. With this mouse model, we demonstrated the potential contribution of NCCs to developing enamel organ and enamel formation, which is different from the results of *Wnt1-Cre/R26R* mice.

MATERIALS & METHODS

Mouse Strains

Mice carrying *Cre* recombinase driven by the protein zero promoter (*P0-Cre*) [24] were mated with *ROSA26R* mice (*R26R*) [20] to generate *P0-Cre/R26R* mice. Embryos were harvested at embryonic day (E) 9.5, E11.5, E12.5, E13.5, E14.5, E16.5, E18.5, and newborn (NB). *P0-Cre* mice were also crossed with *lacZ/EGFP* reporter mice (*Z/EG*) [27], and *P0-Cre/ZEG* double transgenic mouse embryos were obtained at E14.5. Genotypes of the embryos were confirmed with PCR. Animal protocols were reviewed and approved by the University of Michigan Institutional Animal Care and Use Committee.

Tissue Preparation and Immunohistochemistry

Embryos fixed with 4% paraformaldehyde-PBS (PFA) were infiltrated with 20% sucrose and embedded in OCT/Tissue Tek (Sakura Finetek). Serial coronal sections with a thickness of 10 μ m were made and then blocked with 5% sheep serum (S-22, Chemicon) for 30 min. Primary antibodies against the proteins were applied: β -Galactosidase (1:500, ab9361, Abcam), EGFP (1:100, ab13970, Abcam), *Cre* recombinase (1:500, MAB3120, Millipore), p75 (1:200, AB-N01AP, ATS), AP2 (1:25, 3B5, Hybridoma Bank), Sox9 (1:100, sc-20095, Santa Cruz), Cytokeratin 5 (1:500, PRB-160P, Convance), Ki-67 (1:50, M7249, DAKO),

p21 (1:50, 556430, BD Biosciences), and Amelogenin (1:500, gift from Dr. Jan Hu). After overnight primary antibody incubation at 4°C, sections were washed and then incubated for 30 min at room temperature in solutions containing appropriate secondary antibodies, conjugated with Alexa Fluor 488 or Alexa Fluor 594 (1:500, Invitrogen). Sections were then mounted with Gold antifade medium with DAPI (Invitrogen), and examined under Olympus BX51 microscope.

RESULTS

β-Gal Positive Cells are Detected not only in Dental Mesenchyme but also in Enamel Organ

To assess the contribution of NCCs to tooth development, mice carrying both *P0-Cre* and *ROSA26R* (*P0-Cre*(+);*R26R*/+, *P0-Cre/R26R* hereafter) were generated [20; 24]. Once *P0-Cre* commences to express in migrating NCCs, the β-galactosidase (β-gal) indelibly marks the cells, allowing for tracing the NCC lineage by detecting β-gal. The immunostaining results revealed that β-gal⁺ cells were mainly distributed over the mesenchyme of the first branchial arch, including the dental mesenchyme condensed around the thickening dental lamina (Fig. 1). Unexpectedly, a few β-gal⁺ cells were also detected in the enamel organ, which is most evident in E14.5, suggesting the potential contribution of NCCs to this lineage (Fig. 1D). We further examined the enamel organs of various odontogenic stages and found that β-gal⁺ cells were consistently observed in the early epithelial thickening of dental placode (E11.5, E12.5), and in the enamel organs of subsequent odontogenic stages: bud (E13.5), cap (E14.5), bell stages (E16.5, E18.5), and newborn (the stage when enamel and dentin start to be deposited at molar cusp tips) (Fig. 1A-G). We confirmed that the β-gal signal cannot be detected in all negative controls, including samples of *P0-Cre*(+);+/+, *P0-Cre*(-);*R26R*/+ and samples with only secondary antibody (Fig. S1A-F and data not shown). We also substantiated this finding by using *P0-Cre* mice crossed with another reporter mouse line, *Z/EG*, in which cells express EGFP after Cre recombination [27]. Consistent with the results from *P0-Cre/R26R* mice, EGFP⁺ cells were found in the enamel organ (Fig. 1H), which excludes the possibilities of potential artifacts from *R26R* reporter mice or β-gal immunohistochemistry. We also re-examined the *lacZ* expression in tooth germs of *Wnt1-Cre/R26R* mouse embryos (E14.5, E18.5), which was previously reported [21]. Consistent with previous results, the β-gal⁺ cells were detected in dental mesenchyme, but not in the enamel organ (Fig. S2A-D), demonstrating that the expression patterns of *P0-Cre* and *Wnt1-Cre* transgenic mice are not identical. Together, our results suggest that NCC-derived cells contribute to the developing enamel organ, which is generally regarded as an ectoderm-derived structure.

Cre Recombinase is Expressed in Migrating NCCs at Early Embryonic Stages but not in Developing Tooth Germs

Discovering the unanticipated expression of *P0-Cre* transgene in developing enamel organs, we performed immunostaining for Cre recombinase to confirm the cell lineage of these β-gal⁺ cells. Cre recombinase was not detected in dental placode at the initial stage of tooth development (E12.5) (Fig. 2A-D). On the other hand, Cre recombinase was detected in migrating NCCs at E9.0 (Fig. 2E-F), suggesting that β-gal⁺ cells in the tooth germ are derived from the cells that produce Cre recombinase at earlier embryonic stages. Next, we examined the co-localization of several NCC-specific markers [28] with Cre recombinase to demonstrate whether these Cre-labeled cells are indeed NCC-derived cells. It is known that NCCs migrate from rhombomeres 1–4 and contribute to the mesenchyme in the first branchial arch including teeth [17]; thus, we focused on migrating NCCs at the level of hindbrain. At E9.5, all Cre-labeled cells were merged with analyzed NCC-specific markers, including p75 (Fig. 2G), SOX9 (Fig. 2H), and AP2 (data not shown). These results indicate that *P0-Cre* is transiently activated and expressed during NCC migration at earlier

embryonic stages, but not ectopically expressed in the enamel organ at later stages, which demonstrates the presence of *P0-Cre* labeled cells exactly reflects the existence of NCC-derived cells in developing enamel organ.

NCC-derived Cells Give Rise to All Four Cell Types of Enamel Organ

To characterize those β -gal⁺ cells in enamel organ, we further examined their epithelial properties by cytokeratin 5 (K5) immunohistochemistry, since *K5* is expressed in dental epithelium, but not in dental mesenchyme [29]. We observed that the majority of the NCC-derived cells in enamel organ were K5⁻, while some of them were K5⁺ at E12.5-E14.5 (Fig. 3A and data not shown). Noticeably, the morphology of some β -gal⁺/K5⁻ cells in enamel organ looks like that of mesenchymal cells, rather than epithelial cells (Fig. 3A). However, at later stage (E16.5-NB), most of the NCC-derived cells were K5⁺, and only a small part of them was K5⁻ (Fig. 3B and data not shown). This heterogeneity of NCC-derived cells in enamel organ might suggest that NCCs contribute to the enamel organ and undergo mesenchymal-epithelial transition in a time-sequential manner.

Furthermore, to know whether the proliferation properties of NCC-derived epithelial cells and other epithelial cells in enamel organ are different, we examined immunoreactivities for a proliferation marker, Ki-67 and a marker for cell cycle arrest, p21. Both cell populations showed similar distribution of Ki-67 and p21, which suggest these two cell populations have similar proliferation properties (Fig. S3A-B).

To further evaluate the significance of these NCC-derived cells in enamel organ, we then examined their cell fates at later stages of enamel formation. We found that NCC-derived cells contribute to all the four cell types in the enamel organ: outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium, during histodifferentiation of tooth development (Fig. 3C,D). Furthermore, immunostaining of amelogenin, a specific marker for secretory-stage ameloblasts [30], demonstrated that some of the NCC-derived cells give rise to functional ameloblasts (Fig. 3E,F), suggesting that NCC-derived cells actively contribute to dental enamel formation.

DISCUSSION

Based on early transplantation studies of fishes and amphibians, it has been generally believed that enamel, which is made by enamel organ epithelium, is an ectodermal derivative, whereas the dentin-pulp complex together with the surrounding supporting tissues originate from NCC-derived mesenchyme [15; 16]. However, the results from those previous experiments may not be directly applicable to mammalian tooth development, since little evidence demonstrated the conservation of tooth developmental process between non-mammals and mammals. Although consistent results with this traditional concept have been shown by using *Wnt1-Cre/ROSA26R* transgenic mice, our results from *P0-Cre/R26R* mice reveal the potential contribution of NCCs to developing enamel organ and enamel formation. Noticeably, a study of NCC contribution to mammalian tooth development by using long-term culture system of rat embryos with DiI labeling seems to support our finding [17]. The study indicated that most of the DiI-labeled cells were present in the condensed dental mesenchyme. However, DiI-labeled cells were apparently also detected in the enamel organ epithelium, which suggested the contribution of NCCs to enamel organ, although the author didn't indicate this unexpected finding in the article [17]. It is a formal possibility that our finding described here is due to the specific property of the *P0* promoter; for example, the insertion site in the genome may influence its specificity. However, we believe this is less likely by following two reasons: First, Cre recombinase is no longer present in the *P0-Cre* labeled cells in dental epithelium after E12.5; second, all of the Cre producing cells in the hindbrain level at E9.0 are also positive for multiple faithful markers

for migrating neural crest cells. Therefore, we believed that the Cre-labeled cells in the dental epithelium are NCC-derived and thus NCC-derived cells contribute to the developing enamel organ that has been thought to be an ectodermal derivative.

Wnt1-Cre transgene and *P0-Cre* transgene are both regarded to be able to delineate the NCC lineage; however, the *Cre* expression patterns of these two transgenes are not completely identical [4; 19; 24]. In general, most of the NCC derivatives, including ventral craniofacial mesenchyme of branchial arches, cranial ganglia, dorsal root ganglia, and developing enteric nervous system, can be marked in both transgenes. Nevertheless, *P0-Cre* was reported to be ectopically expressed in notochord [24], whereas *Wnt1-Cre* was reported to be expressed in CNS structures, such as diencephalon and mesencephalon of the brain, which have been known to be non-NCC derivatives [18; 19]. Together, both of these two transgenes mark some distinct parts, but not all, of the NCC derivatives as well as some non-NCC derivatives. This discrepancy of expression patterns may explain why the result from *P0-Cre/R26R* mice, in our study, shows additional labeled cell populations, compared to the result from *Wnt1-Cre/R26R* mice. Those β -gal⁺ cells found in enamel organ of *P0-Cre/R26R* mice are probably derived from a specific population of NCCs, which is labeled by *P0-Cre*, but not *Wnt1-Cre*. Admittedly, we cannot completely exclude the possibility of ectopic expression of *P0-Cre* transgene, however, we did clearly demonstrate the heterogeneity of cell population in enamel organ epithelium at the early stage of tooth development even prior to the histodifferentiation and morphodifferentiation of enamel organ. Furthermore, because of the discrepancy of expression patterns between different NCC-specific transgenes [19; 22; 23; 24], it is important to compare the results of lineage analyses at least two transgenic lines, for comprehensive evaluation of NCC contributions in any specific organ structure.

In this study, we also demonstrated that the NCC-derived cells in enamel organ were able to give rise to all four cell types in enamel organ and subsequent functional ameloblasts, suggesting that the NCCs contribute to not only development of dentin-pulp complex and periodontium but also enamel formation. Dental pulp stem cells (DPSCs) are multipotent cells isolated from adult tooth pulps, having capacities of self-renewal and multi-lineage differentiation [31]. Numerous investigations have been devoted to stem cell-based tooth engineering and therapeutic potential of tooth regeneration by using DPSCs [32]. Noticeably, based on our findings here, NCCs may contribute to developing enamel organ and enamel formation. Thus, DPSCs, presumably derived from NCCs, may be competent to be induced to differentiate into enamel-forming epithelial apparatus, which significantly potentiates the regeneration of whole functional teeth by using DPSCs.

In summary, in this study, we demonstrated the contribution of NCCs to developing enamel organ and enamel formation. Our findings not only underscore the necessity of reassessment of traditional concepts in odontogenesis but also implicate the potential capacity of DPSCs for bio-engineered teeth and dental regenerative therapy.

Highlights

1. Cre-based cell tracings of neural crest reveal positive cells in dental epithelium.
2. These cells resemble mesenchymal cells and are negative for epithelial markers.
3. All migrating cells positive for Cre are positive for faithful neural crest markers.
4. Cre is not ectopically produced in developing dental epithelium.
5. These suggest potential contribution of neural crest cells into dental epithelium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

NCC	neural crest cell
P0-Cre	Protein zero-Cre
R26R	ROSA26 Cre reporter
DPSC	dental pulp stem cell

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Figure 1.

β -gal⁺ cells in enamel organs of *P0-Cre/R26R* mice (A-G) and EGFP⁺ cells in enamel organs of *P0-Cre/ZEG* mice (H). Immunohistochemical staining with anti- β -gal antibody showed β -gal⁺ cells (staining for green; arrowheads) were detected not only in dental mesenchyme but in epithelial thickening of dental placode (A, E11.5 ; B, E12.5), and enamel organs in sequential odontogenic stages: bud stage (C, E13.5), cap stage (D, E14.5), early bell stage (E, E16.5), late bell stage (F, E18.5), and early secretory stage (G, newborn). Immunohistochemical staining with anti-EGFP antibody also revealed EGFP⁺ cells (staining for green; arrowhead) in the enamel organ of E14.5 *P0-Cre/ZEG* embryo (H).

Figure 2.

Immunohistochemistry for *Cre* expression and NCC-specific marker expression in E12.5 (A-D), E9.0 (E-F), and E9.5 (G-H) *P0-Cre/R26R* embryos. In E12.5 embryo, anti- β -gal (A; staining for green), anti-*Cre* recombinase (B; staining for red), and DAPI (D) staining revealed *Cre* expression was not detected in the β -gal⁺ cells in dental epithelial thickening (arrowheads) (C, superimposition of A and B). In E9.0 embryo, *Cre* expression (staining for red) was apparently detected in migrating NCCs (arrowhead) and NCC-derived cells (arrow) residing in the branchial arch (E-F; DAPI staining for blue). In E9.5 embryo, the *Cre*-expressing cells (G-H, arrowhead; nuclear staining for red) also expressed NCC-specific markers, p75 (G; membrane staining for green) and SOX9 (H; nuclear staining for green; doubly positive cells showed orange nuclei.). Erythrocytes (G-H; green arrow), showed false-positive signals but could be distinguished from NCCs by morphology. NT, neural tube.

Figure 3.

Characterization of β -gal⁺ cells in enamel organs at various stages of tooth development. Double immunostaining for β -gal (staining for green) and cytokeratin-5 (K5, staining for red) revealed most of the β -gal⁺ cells in E14.5 enamel organ were K5⁻, and the morphology of some β -gal⁺ cells looks like that of mesenchymal cells (A). The proportion of β -gal⁺/K5⁺ cells increased in E16.5 enamel organ (B). Moreover, the β -gal⁺ cells could be detected in all four cell types of E18.5 enamel organ: outer enamel epithelium (OEE), stellate reticulum (SR), stratum intermedium (SI), and inner enamel epithelium (IEE) (C, D). In newborn mice, some of the amelogenin⁺ secretory-stage ameloblasts were also β -gal⁺ (arrowheads) (E, F; amelogenin staining for red). Ae, ameloblasts; Od, odontoblasts.