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# Identification and characterization of neural crest-derived cells in adult periodontal ligament of mice

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ABSTRACT

*Objective:* Cells derived from the neural crest (NC) contribute to the development of several adult tissues, including tooth and periodontal tissue. Here, two transgenic lines, *Wnt1-Cre/ZEG* and *P0-Cre/ZEG*, were analysed to determine the fate and distribution of neural crest cells (NCCs) in adult mouse periodontal ligament (PDL).

*Design:* Paraffin-embedded and decalcified histology samples were prepared from *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice that were 4-, 8-, or 12-weeks old. Expression of GFP (NC-derived cells), NC-markers (Slug, AP-2 alpha, HNK-1, p75NTR and Nestin), and mesenchymal stem cell markers (CD29 and STRO-1) were examined using immunohistochemistry.

*Results:* In four-week-old *Wnt1-Cre/ZEG* mice, $GFP^{(+)}$ NC-derived cells were specifically detected in the mid-zone of PDL. The $GFP^{(+)}$ cells constituted 1.4% of all cells in PDL, and this percentage decreased as the mice aged. The distribution and prevalence of $GFP^{(+)}$ cells were comparable between *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice. NC-$marker^{(+)}$ cells were expressed only in $GFP^{(+)}$ cells while MSC markers were detected only in $GFP^{(-)}$ cells.

*Conclusion:* The prevalence and specific distribution of NC-derived cells in adult PDL of *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mouse were examined. Interestingly, various NC markers, including markers for undifferentiated NCCs, were still expressed at high levels in $GFP^{(+)}$ cells. These observations may indicate that labelled cells in the *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice did not constituted all NC-derived cells, but rather an interesting subset of NC-derived cells. These findings may be useful in understanding the homeostatic character of the PDL and contribute to establishing successful periodontal tissue maintenance.



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*Abbreviations:* NC, neural crest; NCC, neural crest cell; PDL, periodontal ligament; GFP, green fluorescent protein; MSC, mesenchymal stem cell.

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## 1. Introduction

Periodontal ligament (PDL) is a unique connective tissue that anchors two specialized mineralized tissues, tooth cementum and alveolar bone. Development of the PDL initiates shortly after cementoblasts and fibroblasts form cementum. The cementoblasts and PDL cells originate from the inner layer of dental follicles, which comprise cells of ectomesenchyme origin; thus individual cells are derived from either mesenchyme or the neural crest (NC). Therefore, both cementoblasts and PDL cells are, at least in part, derived from NC. Neural crest cells (NCCs) originate at the neural plate border in the ectodermal and neuroectodermal junction during an early embryonic stage. Cranial NCCs emigrate from NC during the neural tube formation, 4-somite to 14-somite stage in mice. In mammals, cranial NCCs migrate laterally and ventrally to the craniofacial region and contribute to tooth development.[1,2] The NCCs are originally multipotent cells, and their fate is determined during migration to their final destination.[3] Recent studies reveal that some NCCs maintain their multipotency even after migration. In fact, undifferentiated multipotent NCCs have been isolated from several NC-derived tissues, including intestine,[4] hair follicle,[5] skin,[6,7] heart,[8] and cornea,[9] and these populations of NCCs play significant roles in the respective tissues as multipotent stem cells.

Understanding the developmental origin of tissue is vital to ensure proper tissue regeneration or reconstruction. Due to the importance of the NC in tissue development, the NC is considered a fourth germ layer; therefore, the fate of NCCs has been investigated. Expression of NC markers is generally stage-specific or transient; consequently, it has been difficult to trace derivatives of the NC using only cell surface markers. To study the fate of NCCs, quail-chick transplantation chimaeras[10] and DiI labelling system[2] have been developed and utilized. Recently, transgenic mice carrying individual Cre recombinase genes driven by tissue specific promoters and floxed-reporter constructs have been used to genetically mark NCCs for lineage-tracing studies. A proto-oncogene, *Wnt1*, is expressed during development of the central nervous system, and the *Wnt1* is expressed in NCCs.[11] Therefore, *Wnt1-Cre* transgenic mice[12] that express Cre-recombinase under the control of the *Wnt1* promoter are used to induce Cre-loxP recombination in a NC-specific manner. The *Wnt1-Cre* line, together with a Cre reporter line *R26R*,[13] have been widely used to trace NCCs, and these tracing studies show that NCCs contribute to the formation of dental mesenchyme in tooth development[1]; such findings are consistent with the classical observations.[2,10] In *Wnt1-Cre/R26R* mice, the majority of PDL cells are shown to be NC derivative.[1] However, the number of non-NC-derived cells increases as tooth development advances.[1,14] Currently, several systems for tracing NCCs during development are available; these include transgenic systems and reporter systems; notably, the findings from studies using different systems are not identical.[12,15–18] Although, the *Wnt1-Cre/R26R* mice are often used to trace NC-derivatives, it is necessary to use different NCC tracing systems to confirm that particular findings are reliable. Thus, we also used a line of *P0-Cre* transgenic mice; in this line, Cre expression is driven by the promoter of the *protein 0* (*P0*) gene, which encodes a cell adhesion molecule in the immunoglobulin superfamily that is specific for NCCs,[9,19] to analyse the presence and distribution of NC derivatives in the adult PDL. As a reporter, we used a *ZEG* Cre reporter line,[20] these mice carry a loxP-flanked *LacZ*, followed by *enhanced green fluorescent protein (GFP)*. Consequently, in mice carrying both *Wnt1-Cre* (or *P0-Cre*) and *ZEG*, NC-derived cells are genetically labelled with GFP, allowing detection of the NC lineage throughout embryogenesis and adulthood.[21–23]

Here, we examined the distribution of $GFP^{(+)}$ NCC derivatives in the adult PDL using *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice. The distribution of $GFP^{(+)}$ cells was similar in *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice, but this distribution differed from distribution of $GFP^{(+)}$ cells in *Wnt1-Cre/R26R* mice. The $GFP^{(+)}$ cells of the PDL were further characterized by assessing the expression of markers for NCCs. Surprisingly, the $GFP^{(+)}$ cells expressed high levels of various NC markers, and these levels were higher than those seen in $GFP^{(-)}$ cells in the PDL of *Wnt1-Cre/ZEG* mice. Our results demonstrated that the *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice labelled almost identical subsets of NC-derived cells in mice, but they did not label all NC-derived cells.

## 2. Materials and methods

### 2.1. Animals and tissue preparation

Two transgenic mouse lines, *Wnt1-Cre*[12] and *P0-Cre*,[15] were separately crossed with *lacZ/EGFP* (*ZEG*) reporter mice.[20] Genotypes of the transgenic animals were determined using polymerase chain reaction (PCR) assays. Genomic DNA was isolated from ear biopsies. The 5′ and 3′ primers used to detect the *Cre* gene were 5′-CGAACATCTTCAGGTTCTGCGG-3′ and 5′-GTCGATGCAACGAGTGATGAGG-3′, respectively (target size 169 bp), and primers for the *ZEG* gene were 5′-GTTCATCTG-CACCACCGGC-3′ and 5′-TTGTGCCCCAGGATGTTGC-3′ (target size 284 bp). All mouse experiments were performed in accordance with the National Institute of Environmental Health Sciences (NIEHS) guidelines regarding the humane care and use of animals in research.

*Wnt1-Cre/ZEG* or *P0-Cre/ZEG* mice that were 4-, 8-, or 12 weeks old were euthanized, and the maxilla, including molars and surrounding tissues, were dissected. Mice that carried only *ZEG* were used as negative controls. The tissue samples were fixed with 4% formaldehyde, decalcified with 10% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin using standard protocols reported elsewhere.[24] Sagittal or axial sections (5 μm thick) were prepared and subjected to the following analysis.

The primary and secondary antibodies used in this study are shown in Table 1. To detect the $GFP^{(+)}$ NCCs, immunohistochemistry was performed using the avidin–biotin complex method. Tissue sections were deparaffinized and treated with 10 mM citric acid buffer (pH 6.0) for antigen retrieval. Endogenous horseradish peroxidase (HRP) was quenched with 3% hydrogen peroxidase ($H_2O_2$), and specimens were then incubated overnight with rabbit anti-GFP antibody, washed several times with PBS, and then incubated with biotinylated anti-rabbit IgG for 30 min. After several washes