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 analyzed 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,

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including markers for undifferentiated NCCs, were still expressed at high levels in GFP[(+)] cells. These observations may indicate that labeled 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.

Keywords

Neural crest cells; Neural crest marker; Periodontal ligament

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 chimeras (10) and DiI labeling 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 analyze 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 L*acZ* followed by *enhanced green fluorescent protein (GFP.* Consequently, in mice carrying both *Wnt1-Cre* (or *P0-Cre*) and *ZEG* NC-derived cells are genetically labeled 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 labeled almost identical subsets of NC-derived cells in mice, but they did not label all NC-derived cells

MATERIALS & METHODS

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'-GTTCATCTGCACCACCGGC-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 were 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 with PBS, samples were incubated with avidin-biotin-HRP mixture for 30 min, and the immunoreactivity was visualized by 3, 3' diamino benzidine tetrahydrochloride (DAB; Vector Laboratories). Hematoxylin was used for counter-staining.

To analyze the expression of NC markers, mesenchymal stem cell (MSC) markers or a cell proliferation marker, in GFP$^{(+)}$ cells double immunofluorescence staining was performed. Following antigen retrieval as described above, sections were incubated with anti-GFP antibody together with one other antibody that is specific to NC cells (i.e., anti-Slug, anti-

AP-2 alpha, anti-HNK-1, anti-p75NTR, or anti-Nestin antibody), MSC markers (anti-CD29 or anti-STRO-1), or a cell proliferation marker (phospho-Histone H3). The immunoreactivity was visualized by fluorescence conjugated secondary anitbodies. The sections without incubated with primary antibodies were served as negative control. Fluorescently labeled sections were mounted in Prolong Gold antifade reagent containing DAPI (Molecular Probes) and observed under a fluorescence microscope; digital images were captured using CCD image capture (DP-70, Olympus, Tokyo, Japan).

Paraffin-embedded samples (n=3) were prepared from each mouse strain (n=3), and nine sections were analyzed in each experiment. The medial and distal sides of the PDL in the medial root of the first maxillary molar were selected as the observation area in the present study. The numbers of immuno-positive cells and total cells within the observation area were counted, and the percentage of immuno-positive cells within the total cell population was calculated. Statistical analyses were performed using Student's t-test, and a p value less than 0.05 was considered significant.

RESULTS

Distribution of GFP[(+)] cells in the PDL of Wnt1-Cre/ZEG mice

To analyze the distribution of GFP[(+)] NCCs in PDL of *Wnt1-Cre/ZEG* mice, we chose 4-weekold mice because root formation is complete by that age. The PDL of the upper first maxillary molar medial root was chosen for sagittal observation, and distal root was chosen for axial observation (Fig. 1a). In *Wnt1-Cre/ZEG* transgenic mice, GFP[(+)] cells were detected in the mid-zone of the PDL between bone and cementum, and some were also seen at the surface of bone (Fig. 1b, white arrowhead) and cementum (Fig. 1c, white arrowhead). More GFP[(+)] cells were evident in the apical region of the PDL than in the cervical region(Fig. 2a). The GFP[(+)] cells on the bone surface were found only in the apical region of the PDL. GFP[(+)] cells constituted only 1.4±0.4% of all cells in the PDL of the medial root in *Wnt1-Cre/ZEG* mice (Fig. 2b).

In contrast to previous reports, we found that the distribution of GFP[(+)] cells in *Wnt1-Cre/ZEG* mice was not identical to that in *Wnt1-Cre/R26R* mice (1, 14). To confirm this observation, we crossed mice that carry a different NC-specific Cre transgene, *P0-Cre* (9, 19), with *ZEG* reporter mice. In the resulting *P0-Cre/ZEG* mice, the population and distribution of GFP[(+)] cells were comparable to those in *Wnt1-Cre/ZEG* mice (Fig. 1d, 2b). We used two different antibodies against GFP and obtained the same results using both antibodies (data not shown). We also confirmed that no GFP[(+)] cells were detected in mice that were designated the negative control because they carried only ZEG (Fig. 1e, 2b). Ubiquitous expression of β-gal[(+)] cells was confirmed (Fig. 1f) in the PDL of *ZEG* reporter mice, verifying that the *ZEG* reporter system was working properly in the tissue and stage we examined. These observations confirmed that the unique expression pattern of GFP[(+)] cells found in the PDL of *Wnt1-Cre/ZEG* mice was not due to the silencing of the *ZEG* reporter, rather the observations indicated that the *ZEG* reporter specifically labeled a subpopulation of NC-derived tissues. A few dendritic-shaped GFP[(+)] cells, which were presumably fully differentiated peripheral nerve cells, were observed in the PDL of *Wnt1-Cre/ZEG* Fig. 1g) and *P0-Cre/ZEG* mice (not shown). The GFP[(+)] cells were also found in odontoblasts in both *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice (Fig. 1h, i), but not in *ZEG* mice (Fig. 1j). The distribution of GFP[(+)] cells and the GFP[(+)] cell/total cell ratio in *Wnt1-Cre/ZEG* mice were similar to those in *P0-Cre/ZEG* mice; therefore, we used only *Wnt1-Cre/ZEG* mice for the subsequent analyses.

Reportedly, the number of non-NC-derived cells in the PDL of *Wnt1-Cre/R26R* mice increases as tooth development advanced (1, 14). It is known that the second molar of

rodents develops later than the first molar. Consequently, even in the same histology sample, the PDL of second molar is at the earlier stage of development compared to that of first molar. The percentage of GFP[(+)] cells in the PDL of second molar was 13.6±1.0%, that was significantly higher than that of first molar (Fig. 2c). Furthermore, to access the age-related differences in the number of NC-derived cells in the PDL of *Wnt1-Cre/ZEG* mice, the numbers of GFP[(+)] NC-derived cells in 4-, 8- and 12-week-old mice were analyzed. The percentage of GFP[(+)] cells decreased with age. There were significantly more GFP[(+)] cells at 4 weeks than at 8 weeks (1.1±0.3%) or at 12 weeks (1.0±0.4%) than at 4 weeks (Fig. 2c).

Expression of NC markers in the PDL of Wnt1-Cre/ZEG mice

To characterize the GFP[(+)] cells in the PDL of *Wnt1-Cre/ZEG* mice, expression of NC-markers (i.e., Slug, AP-2, HNK-1, p75NTR, or Nestin) was examined by immunofluorescence staining (Fig. 3ae). All NC markers examined were detected within PDL cells. Each NC-marker was evident in less than 0.02% of all PDL cells. However, NC-marker signal was evident in many GFP[(+)] cells; specifically, 21.6±6.1% of GFP[(+)] cells were Slug[(+)]32.3±5.1% were AP-2 alpha[(+)]22.0±2.8% were HNK-1[(+)]21.6±2.1% were p75NTR[(+)]and 15.6±3.1% were Nestin[(+)] (Fig. 3g). The sections without incubated with primary antibodies did not show any signal except DAPI (Fig.3f) confirming the specificity of primary antibodies used in this study. Virtually, no cells that expressed a NC marker was also GFP[(−)]cells. At the developmental stage, the NC markers, such as Slug and AP-2 are expressing in NCCs. However the expression of such markers tends to fade as development progresses and very limited numbers of cells keep expressions of these NCC markers in adults. This is the reason why we need to use a genetically labeled NC traceable system to identify NCC-derived cells in adults. Our data showing that part of GFP[(+)] cells in *Wnt1-Cre/ZEG* line are still expressing NC markers while GFP[(−)] cells are not. It may suggest that, GFP[(+)] cells in adult *Wnt1-Cre/ZEG* mice still maintain the characteristics of NCCs at their developmental stage.

Expression of MSC markers and a cell proliferation marker in the PDL of Wnt1-Cre/ZEG mice

To determine whether the GFP[(+)]NC-derived cells remained stem cells, the expression of well-characterized MSC markers, CD29 and STRO-1, was examined. Among the PDL cells, CD29[(+)] or STRO-1[(+)] cells were found at 0.4±0.12% and 0.4±0.27% respectively. Surprisingly, the MSC marker[(+)] cellswere all GFP[(−)]; none were GFP[(+)] (Fig. 4a). The MSC marker[(+)] signal indicated that MSCs were located to the peri-vascular in the PDL, as reported previously (25). The red blood cells in the blood vessels were also detected, however these signals are false positive due to their auto-fluorescence property.

To evaluate the cellular proliferative potential of GFP[(+)] cells in the PDL of *Wnt1-Cre/ZEG* mice, expression of phospho-Histone H3 (Ser10), which is a cell proliferation marker that specifically labels cells in mitosis (M)-phase (26), was analyzed using immunofluorescence staining. Cells with phospho-Histone H3 signal constituted 11.4±1.5% of all GFP[(+)] cells and 0.3±0.2% of all GFP[(−)] cells (Fig. 4b). This observation also confirmed the notion that the GFP[(+)] cells in the PDL of *Wnt1-Cre/ZEG* mice represented a distinct subset of NCCs.

DISCUSSION

Here, we have identified the populations and evaluated the distributions of GFP[(+)]NC-derived cells in the PDL of 4-, 8-, and 12-week-old *Wnt1-Cre/ZEG* mice. The number of labeled NC-derived cells in the PDL of *Wnt1-Cre/ZEG* mice was lower than that in the PDL of *Wnt1-Cre/R26R* mice, which have been analyzed many times. In *Wnt1-Cre/R26R* mice, a majority of PDL cells are labeled as NC derivatives (1, 14, 23). *Wnt1-Cre/ZEG* constructs

may not label all NC-derived cells in the PDL, but rather they may label only a subpopulation. Consequently, we confirmed the relevancy of the NCtraceable system, which we used, using two different GFP antibodies and two *Cre* systems that are driven by different NC-specific promoters. Successful expression of the *ZEG* transgene in a majority of PDL cells was also verified by immuno-detection of β-gal.

As reported previously, the labeling efficiency of the ZEG system becomes low in adult tissues (27–29), and the increased methylation that is evident in older mice may affect Cre-dependent recombination (27). Thus, it is a formal possibility that the entire NC-derivatives may not labeled in the PDL of *Wnt1-Cre/ZEG* mice due to the low labeling efficiency of *ZEG* system in adult. Because of this, it is difficult to exclude a possibility that some of the CD29$^{(+)}$ or STRO-1$^{(+)}$ cells are neural crest origin. However, an interesting result is that a substantial part of GFP$^{(+)}$ cells in the PDL of *Wnt1-Cre/ZEG* mice expressed multiple NC markers while we do not observe any of these NC markers in the GFP$^{(-)}$ cell population. The *Wnt1-Cre/ZEG* mice may label a selected population of NC-derived cells in PDL at adult stages that expressed various NC-markers, including markers expressed in undifferentiated NCCs.

Previously, cells expressing markers of multipotency and NC markers (e.g., HNK-1, p75NTR, and Nestin) were isolated from neurosphere culture PDL cells (30, 31). The neurospheres were able to differentiate into, not only mesenchymal lineages, but also neuronal and mesodermal progeny; these findings indicated the presence of undifferentiated NC-derived cells. Moreover, PDL cell-derived neurospheres reportedly forms from less than 0.01% of all cells in established culture (31). Similarly, we have found that 0.01~0.02% of all PDL cells *in vivo* expressed markers of undifferentiated NCCs. These data indicated that only a small population of NC-derived cells in PDL may maintain the undifferentiated nature of NCCs.

Interestingly, the GFP$^{(+)}$ cells in PDL of *Wnt1-Cre/ZEG* mice did not express CD29 or STRO-1, which are well-known MSC markers. Various types of stem cells have been isolated from periodontal tissue, such as stem cells from exfoliated deciduous teeth (SHED) (32), dental pulp stem cells (DPSCs)(33), periodontal ligament stem cells (PDLSCs) (25) etc. These are characterized as MSCs based on three criteria: they have colony-forming ability, they have multi-differentiating potency, and they express MSC markers—including CD29 (34, 35) and STRO-1 (25, 36–38). We found that only 0.4% of the cells in the PDL of *Wnt1-Cre/ZEG* mice were either CD29$^{(+)}$ or STRO-1$^{(+)}$ cells, and none of these cells were GFP$^{(+)}$ cells. Previous studies clearly demonstrate the substantial contribution of NC-derived cells during the developmental stages of periodontal tissue (1, 39, 40), however, non-NC derived cells become evident as tissue development progresses (1, 14). Recent studies have revealed that bone marrow-derived circulating MSCs are found in peripheral blood vessels and are thought to be delivered to the mesenchymal organs, especially to tissues that undergo rapid remodeling (41). In addition, blood vessel-associated pericytes showed similar characteristics as MSC (42). The MSCs in bone marrow, dental pulp and PDL are reported to be localized at the perivascular region further support the characteristics of pericyte (38). In this study, we found CD29$^{(+)}$ or STRO-1$^{(+)}$ cells, which are GFP$^{(-)}$ and have perivascular localization. Our findings may confirm the presence of pericyte which are non NC derivative and has MSC-like characteristics, in the adult stage of PDL. These results further support our findings that the GFP$^{(+)}$ and NC marker$^{(+)}$ cell population is distinct from previously reported MSCs or pericytes.

To the best of our knowledge, this is the first report to analyze the expression of various NC markers within the adult PDL *in vivo*. An interesting population of NC-derived cells was identified using two transgenic lines, *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice, in which NC-

derived cell can be traced. At this point, we cannot explain the observation that the *Wnt1-Cre/ZEG* system labeled only a select population of NC-derived cells. However, this select population of NC-derived cells expressed various NC-markers (i.e., Slug, AP-2 alpha, HNK-1, p75NTR, and Nestin). Further investigation of this interesting subset of NC-derived cells, including *in vitro* experiments, may be informative and enhance the understanding of the homeostatic property of the PDL that contributes to establish successful periodontal tissue maintenance.

Acknowledgments

Abbreviations

NC	neural crest
NCC	neural crest cell
PDL	periodontal ligament
GFP	green fluorescent protein
MSC	mesenchymal stem cell

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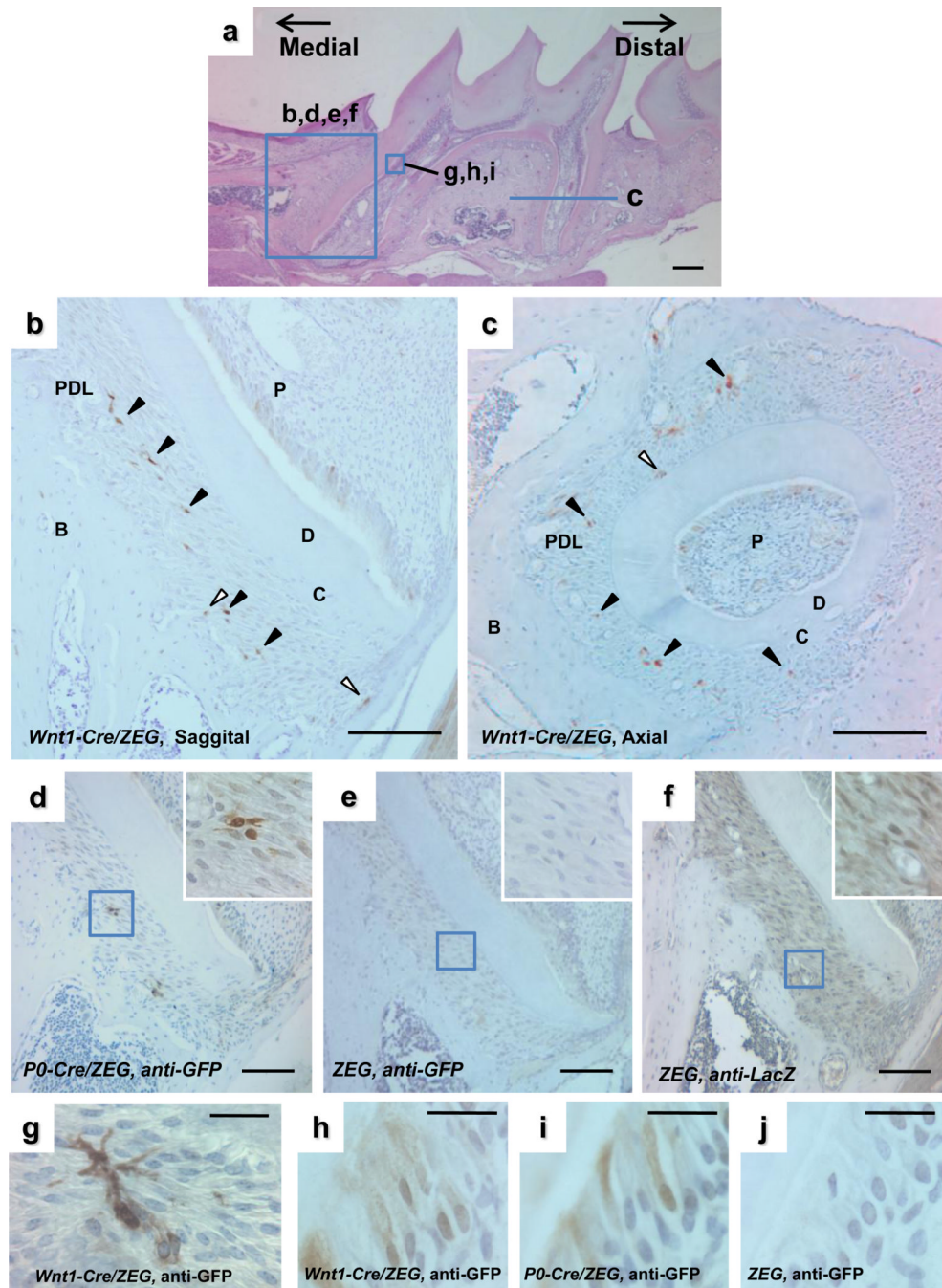
Fig. 1. Distribution of GFP$^{(+)}$ NCCs in the PDL of *Wnt1-Cre/ZEG* mice

Hematoxylin and eosin-stained section shows area under observation which extends from the maxillary first molar (a). Immunohistochemical staining of GFP$^{(+)}$ cells in sagittal (b) and axial (c) sections of the PDL from *Wnt1-Cre/ZEG* mice. The GFP$^{(+)}$ cells (arrowhead) were preferentially distributed in the middle of the PDL, and some were detected at the surface of bone (b, white arrowhead) and cementum (c, white arrowhead). The distribution of GFP$^{(+)}$ cells was similar in *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice (d), but no GFP$^{(+)}$ cells were found in only *ZEG* carrying mice (e). The ubiquitous distribution of β-gal$^{(+)}$cells suggests that the ZEG reporter locus was active in these cells (f). Dendritic-shaped differentiated peripheral nerve cells were detected in the PDL of *Wnt1-Cre/ZEG* mice (g).

GFP$^{(+)}$ cells were also found in odontoblasts in *Wnt1-Cre/ZEG* (h) and *P0-Cre/ZEG* mice (i), but not in *ZEG* mice (j). B: bone; C: cementum; D: dentin; P: pulp. Bar: 100 μm.
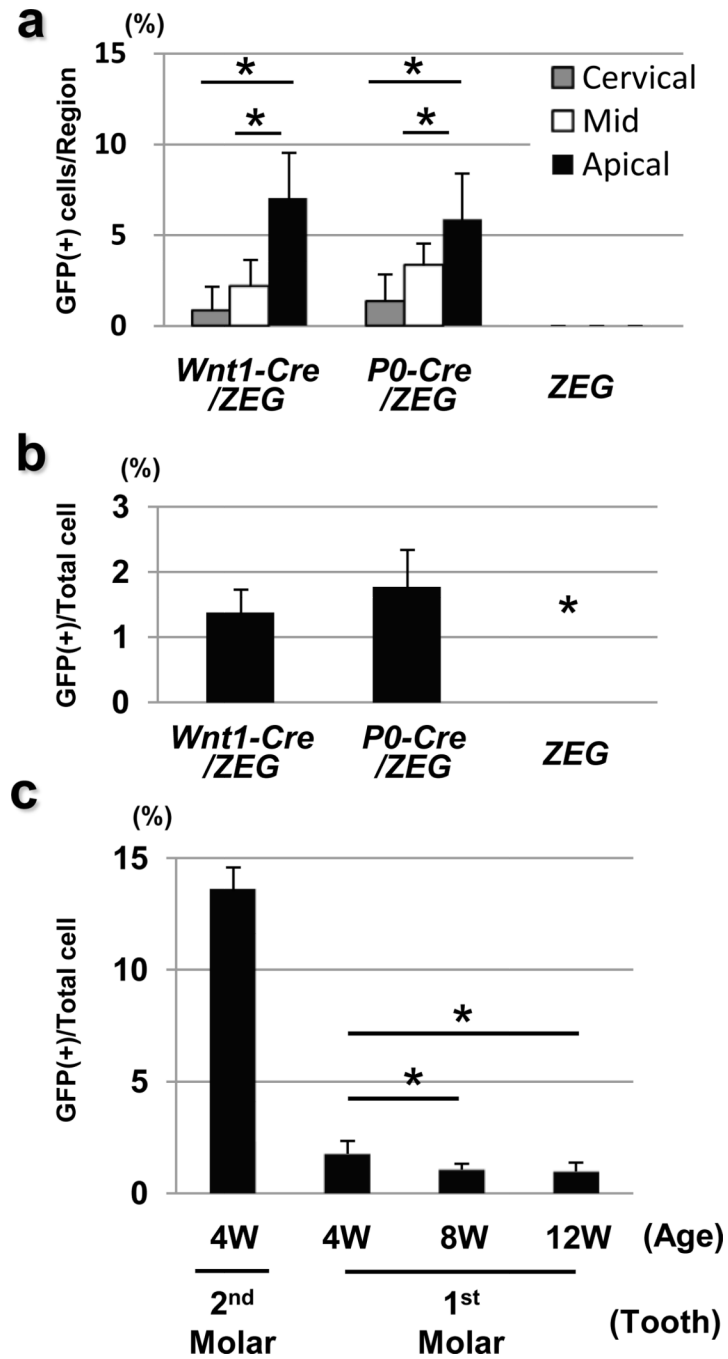
Fig. 2.
The distribution of GFP$^{(+)}$ cells in the different region of PDL in Wnt1-Cre/ZEG mice was compared (a). Apical part of PDL has the most abundant distribution of GFP$^{(+)}$ cells. The number of GFP$^{(+)}$ cells in the PDL of *Wnt1-Cre/ZEG* and *P0-Cre/ZEG* mice was comparable but the GFP$^{(+)}$ was not detected on ZEG mice (b). The percentage of GFP$^{(+)}$ NC-derived cells in the PDL of different tooth and different age was compared. The GFP$^{(+)}$ cell ratio was significantly higher in the PDL of 2nd molar compared with first molar. In the PDL of first molar the GFP$^{(+)}$ cell ratio in PDL decreased with age (c) *: Statistical significance p<0.05.
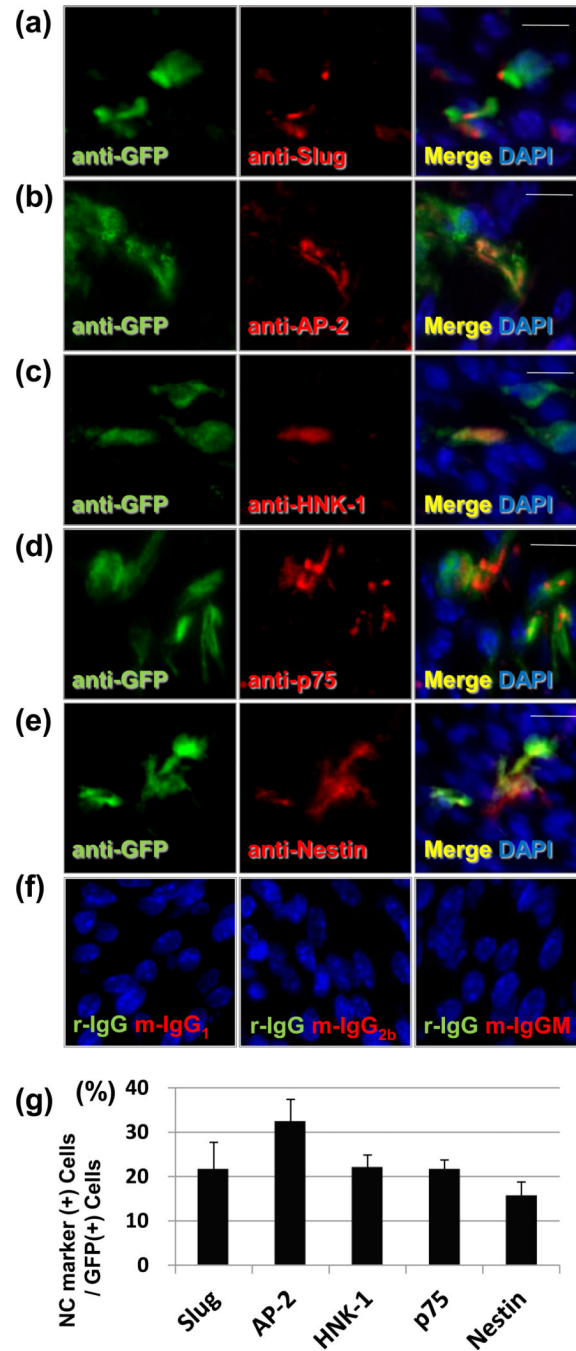
Fig. 3. Expression of neural crest (NC) markers in the PDL of *Wnt1-Cre/ZEG* mice
Immunofluorescence staining to identify NC markers among GFP[(+)] NCCs in adult PDL cells is shown (a-e). No-signal was detected when samples were reacted without primary antibodies (f). The percentage of NC marker[(+)] cells among all GFP[(+)] cells was 21.6±6.1% for Slug, 32.3±5.1% for AP-2, 22.0±2.8% for HNK-1, 21.6±2.1% for p75, and 15.6±3.1% for Nestin (g). Bar: 20 μm.
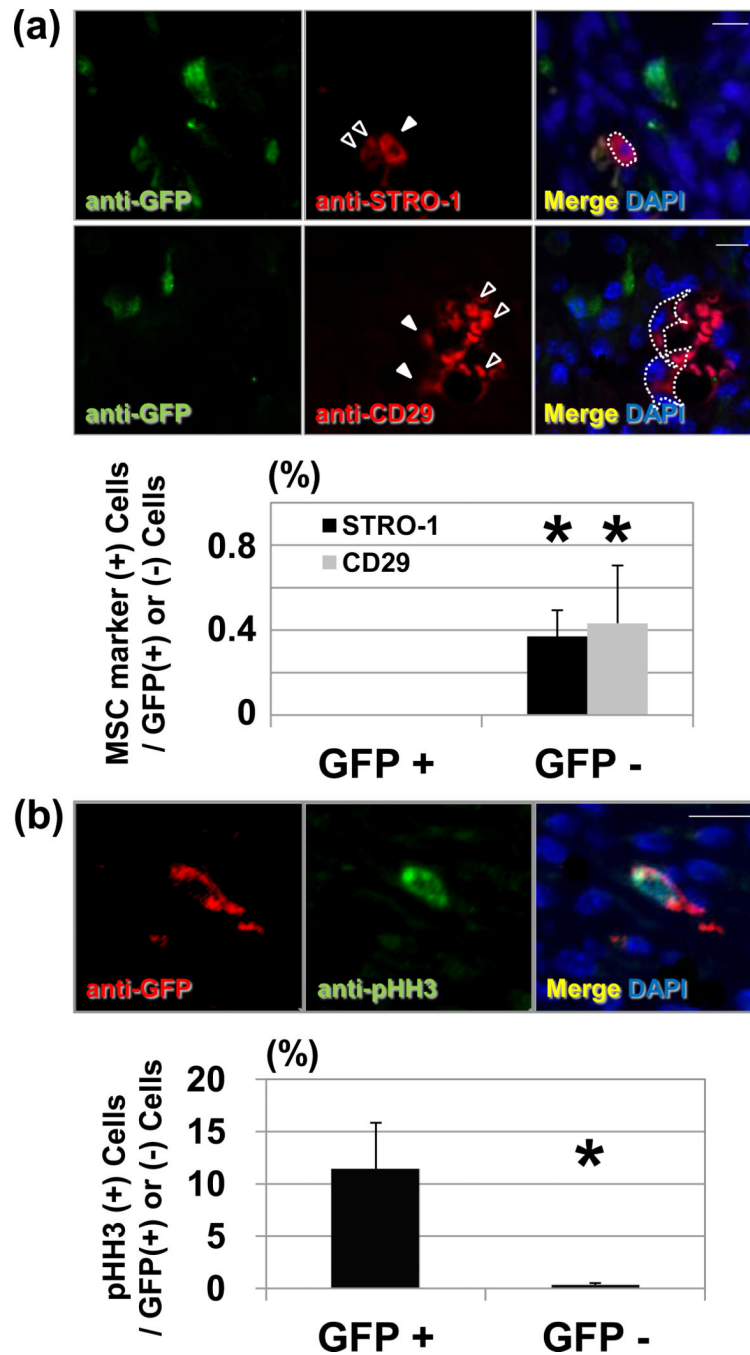
Fig. 4. Expression of mesenchymal stem cell (MSC) markers and a cell proliferation marker in the PDL of *Wnt1-Cre/ZEG* mice

Immunofluorescence staining of CD29 and STRO-1 on PDL cells (a). CD29 or STRO-1[(+)] cells were found only on GFP[(−)] cells and showed perivascular localization (arrowheads). Red blood cells are detected due to their autofluorescence property (open arrowheads). The expression of phospho-Histone H3 (pHH3)[(+)] cells among GFP[(+)] NCCs and GFP[(−)] cells were shown (b). GFP[(+)] cells showed significantly higher cell proliferation in comparison to GFP[(−)] cells. Bar: 20 μm. *: Statistical significance p<0.05.

Table 1

Primary and secondary antibodies used in this study.

Antibody Name	Clone No.	Manufacturer/Distributer	Dilution
rabbit anti-GFP	A11122	Molecular Probes (Carlsbad, CA)	1:100
chicken anti-GFP	ab13970	abcam (Cambridge, MA)	1:200
biotinylated anti-β-galactosidase	G1041-42	US Biological (Massachusetts, MA)	1:100
mouse anti-Slug	62.1E6	DSHB	1:100
mouse anti-AP-2 alpha	3B5	DSHB	1:100
mouse anti-Nestin	Rat-401	DSHB	1:100
mouse anti-STRO-1	STRO-1	DSHB	1:100
mouse anti-HNK-1	VC1.1	Sigma-Aldrich (St. Louis, MO)	1:100
mouse anti-p75	ME20.4	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)	1:50
mouse anti-CD29	18/CD29	BD Bioscience (Franklin Lakes, NJ)	1:100
rabbit anti-phospho-histone H3	Ser10	Millipore (Temecula, CA)	1:100
Alexa Fluor 488 anti-rabbit IgG	n/a	Molecular Probes (Carlsbad, CA)	1:100
Alexa Fluor 488 anti-chicken IgG	n/a	Molecular Probes (Carlsbad, CA)	1:100
Alexa Fluor 594 anti-mouse IgG$_1$	n/a	Molecular Probes (Carlsbad, CA)	1:100
Alexa Fluor 568 anti-mouse IgG$_{2b}$	n/a	Molecular Probes (Carlsbad, CA)	1:100
Alexa Fluor 568 anti-mouse IgM	n/a	Molecular Probes (Carlsbad, CA)	1:100
biotinylated anti-rabbit IgG	n/a	Vector Laboratories (Burlingame, CA)	1:200
biotinylated anti-chiken IgG	n/a	Vector Laboratories (Burlingame, CA)	1:200

DSHB; Developmental Studies Hybridoma Bank, Department of Biology, The University of Iowa