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Localization and osteoblastic differentiation potential of neural crest-derived cells in oral tissues of adult mice

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ABSTRACT

In embryos, neural crest cells emerge from the dorsal region of the fusing neural tube and migrate throughout tissues to differentiate into various types of cells including osteoblasts. In adults, subsets of neural crest-derived cells (NCDCs) reside as stem cells and are considered to be useful cell sources for regenerative medicine strategies. Numerous studies have suggested that stem cells with a neural crest origin persist into adulthood, especially those within the mammalian craniofacial compartment. However, their distribution as well as capacity to differentiate into osteoblasts in adults is not fully understood. To analyze the precise distribution and characteristics of NCDCs in adult oral tissues, we utilized an established line of double transgenic (P0-Cre/CAG-CAT-EGFP) mice in which NCDCs express green fluorescent protein (GFP) throughout their life. GFP-positive cells were scattered like islands throughout tissues of the palate, gingiva, tongue, and buccal mucosa in adult mice, with those isolated from the latter shown to form spheres, typical cell clusters composed of stem cells, under low-adherent conditions. Furthermore, GFP-positive cells had markedly increased alkaline phosphatase (a marker enzyme of osteoblast differentiation) activity and mineralization as shown by alizarin red staining, in the presence of bone morphogenetic protein (BMP)-2. These results suggest that NCDCs reside in various adult oral tissues and possess potential to differentiate into osteoblastic cells. NCDCs in adults may be a useful cell source for bone regeneration strategies.

1. Introduction

During embryonic development of vertebrates, neural crest cells migrate in a ventro-lateral manner and populate the branchial arches. These ectodermal-derived cells extensively contribute to formation of mesenchymal structures in the head and neck as they migrate [1–3]. Furthermore, they also have a self-renewal capability and potential to differentiate into several different neural crest lineages, including neurons, glial cells, myofibroblasts, melanocytes, adipocytes, chondrocytes, osteoblasts, odontoblasts, and connective tissues [4–6].

Recently, the persistence of such highly pluripotent cells into adulthood has been proposed to provide an explanation for the vast regenerative potential demonstrated by many mammalian craniofacial tissues [7–9]. Interestingly, some neural crest (NC) cells are maintained in an undifferentiated state as NC-derived cells (NCDCs) throughout the life of the animal [10,11], and NCDCs from the oral or olfactory mucosa, which are easily accessible in the oral and maxillofacial regions, can be cultured [7–9,12] and may be a useful

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cell source for regeneration of those regions. However, previous studies have not revealed the precise distribution of NCDCs in the oral and maxillofacial regions in adulthood, due to the lack of appropriate and reliable markers in adults.

Myelin protein zero (P0) was originally identified as a Schwann cell-specific protein [13] and is expressed in a subpopulation of neural crest cells, as well as in both non-myelinating and myelinating Schwann cells [14,15]. The proto-oncogene *Wnt1* is expressed only during development of the central nervous system. Cranial, spinal ganglia, and skeletogenic neural crest cells in the branchial arches are derived from *Wnt1* expressing precursor cells in the central nervous system [16,17]. Thus, expressions of *P0* and *Wnt1* are useful as markers to follow the migration and differentiation of NCDCs throughout embryogenesis.

Although *P0* and *Wnt1* are the most reliable markers of NCCs in embryos, their expressions are completely silenced before birth. Recently, genetic marking using Cre-recombinase has been applied to long-term tracing of NCDCs in *P0-Cre* and *Wnt1-Cre* mice [18–20]. Those findings prompted us to investigate whether NCDCs obtained from the oral and maxillofacial regions of adult mice could be used for regeneration, and subsequent application in future therapeutic strategies.

In the present study, we utilized *P0-Cre/CAG-CAT-EGFP* mice (P0 mice), in which NCDCs remain labeled with GFP after birth [19,21,22]. We observed the distribution of NCDCs in adult P0 mice and isolated them from other cells with high purity. Our findings demonstrate that GFP-positive NCDCs isolated from adult mice have potential to differentiate into osteoblast-like cells.

2. Materials and methods

2.1. Animals

Transgenic mice expressing the Cre enzyme driven by the P0 promoter were crossed with *CAG-CAT-EGFP* transgenic mice [19,21]. In the *P0-Cre/CAG-CAT-EGFP* double transgenic mice (P0 mice), NCDCs were identified by evaluating GFP expression after P0-Cre-mediated DNA recombination [20]. To examine genotypes, polymerase chain reaction (PCR) analyses for *P0-Cre* and *CAG-CAT-EGFP* were performed as previously described [19,21,22]. All procedures were approved by the Ethical Board for Animal Experiments of Showa University (Approval No. 18089).

2.2. Observation of EGFP-positive cells

The appearance of GFP in adult P0 mouse specimens was examined using fluorescence stereomicroscopy (MVXI10; OLYMPUS, Tokyo, Japan). Frozen tissue sections were fixed with 4% paraformaldehyde and stained with the primary anti-GFP antibody (rabbit polyclonal, Chemicon, Temecula, CA). The sections were then incubated with FITC-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA) and examined with a florescence microscope (BIOREVO BZ-9000; KEYENCE, Osaka, Japan) [9].

2.3. Cell cultures

Cells were isolated from buccal mucosa tissues of adult P0 mice (10–12 weeks old) and incubated in Dulbecco's modified eagle medium (DMEM) containing 1% Dispase II (Godo Shusei Co., Ltd., Tokyo, Japan) for 1 h at 37 °C. After separating epithelium and lamina propria tissues, the lamina propria of buccal mucosa was digested in 0.1% collagenase (WAKO, Osaka, Japan) and 0.2% Dispase II (Godo Shusei Co., Ltd.) in DMEM for 1 h at 37 °C. Cell suspensions from the lamina propria of buccal mucosa were then seeded into αMEM with 10% FBS in type I collagen-coated plates (IWAKI, Chiba, Japan). For sphere-forming cultures, cells were seeded into serum-free sphere-forming medium consisting of DMEM/F-12 (1:1), supplemented with 20 ng/ml epidermal growth factor (EGF; Invitrogen, Carlsbad, NM), 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen), and B27 supplement (Invitrogen), then cultured at 37 °C with 5% CO_2 [23] in non-adhesive 96-well culture plates (CellSeed, Tokyo, Japan) for 7 days.

2.4. Flow cytometric analysis and cell sorting

Cell suspensions from oral mucosa specimens were placed into PBS with 10% FBS, then those at a density of 5×10^5 cells/ml were subjected to cytometric analysis and sorting. For flow cytometry and cell sorting, cells were subjected to a FACSAria II (BD Biosciences, San Diego, CA) using a laser at 488 nm, with a 530/30 band pass filter, 70-μm sort nozzle, and 30.0-kHz drive frequency [24]. Data acquisition and analyses were performed using BD FACSDiva 6.1.2 software, which was gated for a high level of GFP expression. Clear separation of GFP-positive and -negative cells allowed for easy sorting. Sorted GFP-positive and -negative cells were resuspended in sphere-forming medium, and cultured in non-adhesive 96-well culture plates.

2.5. Osteoblastic differentiation

Osteoblastic differentiation was induced by culturing in αMEM with 10% FBS containing 10 mM β-glycerol phosphate, 0.5 mM ascorbic acid, 10^{-8} M dexamethasone (Sigma–Aldrich, St Louis), and 200 ng/ml recombinant human BMP-2 (R&D systems, Minneapolis) for 14 days, with the medium changed every 3 days. Cultured cells were stained for alkaline phosphatase (ALP) using naphthol AS-MX phosphate and fast blue BB salt (Sigma–Aldrich), as previously described [25]. After washing cells with PBS, 40 mM Alixarin red stain (Sigma–Aldrich) was added to the wells and incubated at room temperature for 10 min.

3. Results

3.1. Localization and diversity of GFP-positive cells in buccal mucosa, palate, gingiva, and tongue

First, we observed the distribution of GFP-positive cells, which were considered to have a neural-crest origin, in buccal mucosa, palate, gingiva, and tongue samples obtained from adult P0 mice (Fig. 1A–C). Using fluorescence microscopy, GFP-positive regions appeared as green spots, and were observed in the entire oral mucosa in sagittal and frontal sections of oral tissue (Fig. 1A and B). GFP-positive regions also appeared on the surface of the entire palate in a manner similar to the buccal mucosa (Fig. 1A and B). In addition, GFP-positive regions, noted as green dots, were observed on the surface of the tongue (Fig. 1C). Although GFP-positive regions were found in these locations in tissues obtained from all P0 mice observed (more than 10 mice), the detailed locations and numbers of each spot or dot varied among them (data not shown).

3.2. Observation of GFP-positive cells in periodontal tissues

To elucidate the precise distribution of GFP-positive cells in periodontal tissues, we observed tissue samples under a fluorescence microscope with lower and higher levels of magnification (Fig. 2A and B). GFP-positive spots were found in gingiva around the incisors and molars of the upper and lower jaws (Fig. 2A). However, there were no GFP-positive regions on the surfaces of those teeth. On the surface of the gingiva, GFP-positive spots were found, some of which were located adjacent to teeth (Fig. 2B).

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