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Identification of gene expression profile of neural crest-derived cells isolated from submandibular glands of adult mice

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ABSTRACT

Neural crest cells in the embryo migrate to reach target sites as neural crest-derived cells (NCDCs) where they differentiate into a variety of derivatives. Some NCDCs are maintained in an undifferentiated state throughout the life of the animal and are considered to be a useful cell source for regenerative medicine. However, no established method to obtain NCDCs sufficient for regenerative medicine from adults with high purity has been presented, since their distribution in adult tissues is not fully understood. It is critical to identify reliable markers for NCDCs in adults, as the expressions of *P0* and *Wnt1*, the most reliable NCDC markers, are shut off in the embryonic stage. To analyze the characteristics of NCDCs in adult tissues, we utilized a double transgenic mouse strain, P0-Cre/CAG-CAT-EGFP transgenic mice (P0 mice), in which NCDCs were shown to express EGFP and we were able to recognize GFP-positive cells in those. We focused on the submandibular glands (SMGs), which are known to be derived from the neural crest. GFP-positive cells were shown to be scattered like islands in the SMGs of adult P0 mice. We surgically removed SMGs from adult mice and digested samples into single cell suspensions. GFP-positive cells separated using flow cytometry expressed a high level of *Sox10*, a marker of embryonic neural crest cells, suggesting successful isolation of NCDCs. To identify candidate marker genes in isolated NCDCs, we performed DNA microarray analyses and real-time PCR analysis of GFP-positive and -negative cells isolated from P0 mice, then selected genes showing differential gene expression patterns. As compared to GFP-negative cells, GFP-positive cells expressed *Gpr4* and *Ednrb* at higher levels, whereas *Pdgfra* and *Pdgfrb* were expressed at lower levels. Furthermore, DNA microarray analysis showed that GFP-positive cells were positive for aquaporin 5, a marker for acinar cells. Together, our results indicate that NCDCs in adult SMGs have characteristic gene expression profiles specially their cell surface molecules. Cell sorting using a combination of these specific cell surface proteins would be a useful strategy for isolation of NCDCs from SMGs with high purity.

1. Introduction

In vertebrates, neural crest cells (NCCs) are induced at the dorsolateral edge of the neural plate during early developmental stage [1]. They then undergo an epithelium to mesenchyme transition, become delaminated from the neural tube, and migrate extensively within the embryo as neural crest-derived cells (NCDCs) to reach target sites where they differentiate into a wide range of cell types depending on their origin, including neurons, glial cells, pigment cells, smooth muscle cells of the cardiovascular tissue, teeth, and craniofacial bone tissue and cartilage [2–5]. Some NCDCs are maintained in an undifferentiated state throughout the life of the animal and exist not only in the embryonic neural crest, but also in various neural crest-derived tissues in both fetal and adult stages, where they show multipotent and self-renewing abilities [6–9]. Since those studies showed that NCDCs may exist in various adult tissues, these cells are considered to be a potentially useful source for regenerative medicine and cell transplantation treatment. NCDCs have been isolated from some embryonic tissues [10–12] and shown to express various embryonic neural crest-specific markers [13–16]. However, no effective method for isolating them from adult tissues with high purity has been presented, since

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their specific markers in adult tissues are yet to be identified. Furthermore, their distribution and characteristic gene expression profiles in adult tissues have not been fully elucidated.

Submandibular glands (SMGs) are major salivary glands whose generation is induced by reciprocal epithelial and mesenchymal interactions during embryogenesis [17]. A few studies have reported the existence of NCDCs in embryonic SMGs [5,18,19]. It is known that embryonic mesenchymal tissues in SMGs are derived from cranial NCCs, thus it is considered possible that SMGs have large numbers of NCDCs, because they are neural crest-derived large organs, and may be a particularly useful cell source for regenerative medicine. However, the distributions and characteristics of NCDCs in adult SMGs have not been fully elucidated, and it is not known whether they can be isolated from adult organs. Although *P0* and *Wnt1* are the most reliable markers of NCDCs in embryos, their expressions are completely silenced before birth. Furthermore, even though NCDCs in adults express several NCDC-related marker genes, including *Sox10*, *p75*, and *Snail*, they are also expressed on various types of cells. Therefore, it is very important to determine reliable cell surface molecules that are exclusively expressed on NCDCs in adult tissues. Recently, genetic marking using Cre-recombinase has been applied to long-term tracing of NCDCs in *P0-Cre* and *Wnt1-Cre* mice [20–22].

In the present study, we analyzed the distribution and characteristic gene expression profiles of NCDCs in SMGs of adult *P0-Cre/CAG-CAT-EGFP* mice, in which NCDCs in tissues are labeled with GFP even after birth, in order to establish an effective method for isolating NCDCs from adult tissues [21–23]. Our results showed that cell sorting using several specific cell surface molecules may be a useful method for purification of NCDCs from various adult tissues and serve as a starting point for therapeutic studies.

2. Materials and methods

2.1. Animals

Transgenic mice (Tg) expressing the Cre enzyme driven by the P0 promoter [21] were crossed with *CAG-CAT-EGFP* adult (8–12 weeks old) transgenic mice [22]. In P0-Cre/floxed-EGFP double transgenic mice (P0 mice), neural crest-derived cells were identified by evaluating GFP expression after P0-Cre-mediated DNA recombination [23]. To examine the genotypes, polymerase chain reaction (PCR) analyses for *P0-Cre* and *CAG-CAT-EGFP* were performed as previously described [21,22]. *P0-Cre* recombinase Tg and *CAG-CAT-EGFP* Tg mice were kindly provided by Dr. K. Yamamura (Kumamoto University, Kumamoto, Japan) and Dr. J. Miyazaki (Osaka University, Osaka, Japan), respectively. All procedures were approved by the Ethical Board for Animal Experiments of Showa University (Approval No. 13034).

2.2. Observation of EGFP-positive cells

The appearance of GFP in submandibular glands (SMGs) of adult P0 mice was examined using fluorescence stereomicroscopy (MVXI10; OLYMPUS, Tokyo, Japan).

2.3. Isolation and flow cytometric analysis of salivary gland cells

SMGs were extracted from adult (8–12 weeks old) P0 mice, and dissected and washed in αMEM (Sigma–Aldrich Co., St. Louis, MO), then pieces (1.0 mm^3) of SMGs were incubated in αMEM containing 0.5 mg/ml collagenase A (Roche Diagnostics, Mannheim, Germany) for 60 min at 37 °C. Cell suspensions from SMGs 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, 100-μm sort nozzle, and 30.0-kHz drive frequency, and sterilized with 10% bleach. Data acquisition and analyses were performed using BD FACSDiva 6.1.2 software, gated for a high level of GFP expression. Clear separation of GFP-positive and -negative cells allowed for easy sorting. Sorted cells were pelleted by centrifugation and then used for the following analyses.

2.4. RNA isolation and DNA microarray analysis

RNA was isolated using an RNeasy Plus Kit (Qiagen, Germantown, MD). The concentration and quality of RNA was determined by the Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA Integrity Number (RIN) deduced from this analysis was 7.0–10 for all samples, which denoted excellent RNA quality with no degradation [24]. The final concentrations of total RNA varied from 15 to 20 ng/μl and 150 ng of RNA from each pool was subjected to a single linear amplification labeling reaction with Cy3. RNA was hybridized to Agilent mouse whole genome 44 K microarray slides, using the Agilent one-color gene expression hybridization protocol. Slides were scanned (Agilent G2505B) at a resolution of 5 μm using an extended dynamic range protocol and images were processed with Agilent Feature Extraction software 10.5.1.1 [25]. Microarray results were analyzed using GeneSpring GX 12.0 software (Agilent), according to the workflow presented in the manual, using quantile normalization (percentile shift 75%), filter probesets by

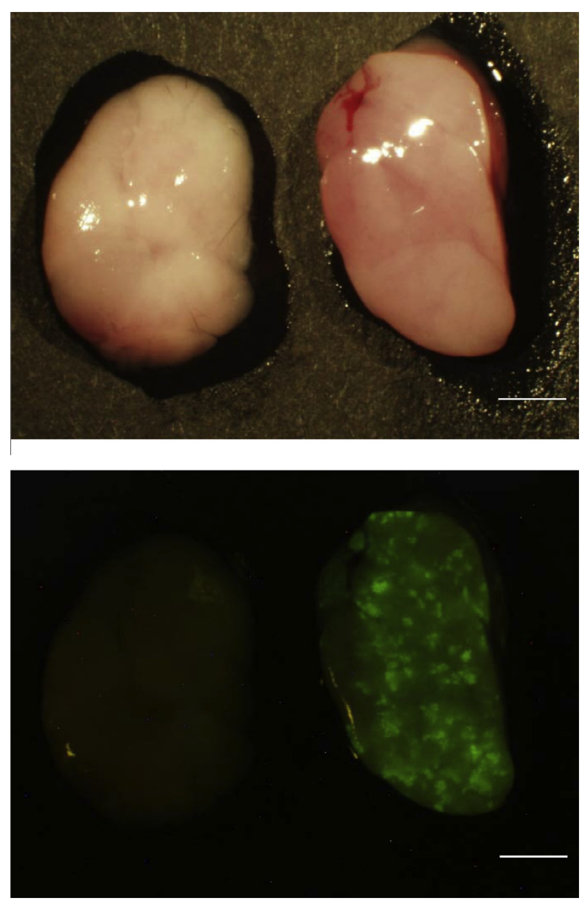

Fig. 1. Localization of NCDCs in SMGs of adult P0 mice. (A, B) Stereoscopic fluorescence microscope images of adult SMGs. Panel A shows a bright-field image and Panel B a corresponding fluorescence image. The surface appearance of representative P0 (right) and wild type (left) mice is shown. GFP labeled cells (green) were observed in SMGs of P0 mice. Scale bar = 10 mm.