Biochemical and Biophysical Research Communications 446 (2014) 481–486

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 18 February 2014
Available online 12 March 2014

Keywords:
Neural crest-derived cells
Adult
Submandibular gland
DNA microarray
Cell surface marker proteins

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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http://dx.doi.org/10.1016/j.bbrc.2014.02.130
0006-291X/

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

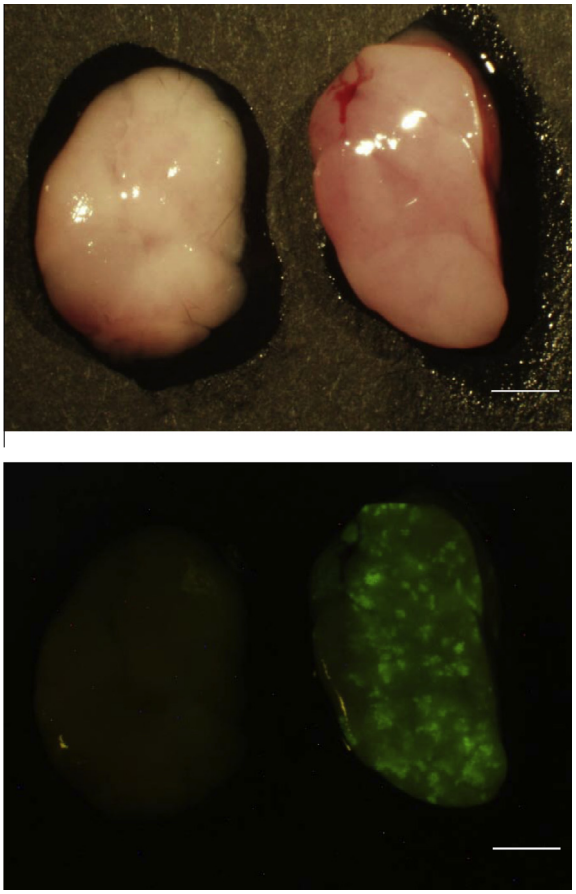

Fig. 1. Localization of NCDCs in SMGs of adult P0 mice. (A, B) Stereoscopic fluorescence microscope images of adult SMGs. Panel A shows a bright-field image and Panel B a corresponding fluorescence image. The surface appearance of representative P0 (right) and wild type (left) mice is shown. GFP labeled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

flags (selected detected and compromised flags), and filter probesets by expression of normalized data (20.0–100.0th percentile).

2.5. Real-time PCR

First-strand cDNA synthesis was carried out with Superscript III reverse transcriptase (Invitrogen Co., Carlsbad, CA) primed with random hexamer. Quantitative PCR reactions were performed using a Taqman based assay with the StepOnePlus detection system, according to the manufacturer's recommendations (Applied Biosystems, Foste City, CA). Reactions were run for 40 cycles. The following Taqman Gene Expression Assay sets were used: Mm01 300162_m1 (*Sox10*), Mm01309638_m1 (*p75*), Mm04208233_g1 (*Twist1*), Mm00441533_g1 (*Snail1*), Mm00432989_m1 (*EDNRB*), Mm00558777_s1 (*GPR4*), Mm00440701_m1 (*Pdgfra*), and Mm04 35546_m1 (*Pdgfrb*). Mm9999915_g1 (*GAPDH*) was used as the endogenous control. All samples were run in triplicate.

2.6. Statistical analysis

Data were analyzed using Student's *t* test, with *p* values less than 0.01 considered to be significant. Each experiment was performed at least twice and representative data are presented as the mean ± S.D. of at least 3 independent replicates.

3. Results and discussion

3.1. Localization of cells derived from neural crest in submandibular glands

To analyze the distribution of NCDCs in adult tissues, we utilized SMGs obtained from postnatal 8-week old adult P0 mice and observed them with a florescence stereomicroscope, as previous studies have shown that embryonic SMG mesenchyme is derived from cranial NCCs in *Wnt1-Cre*/ROSA26 transgenic mice

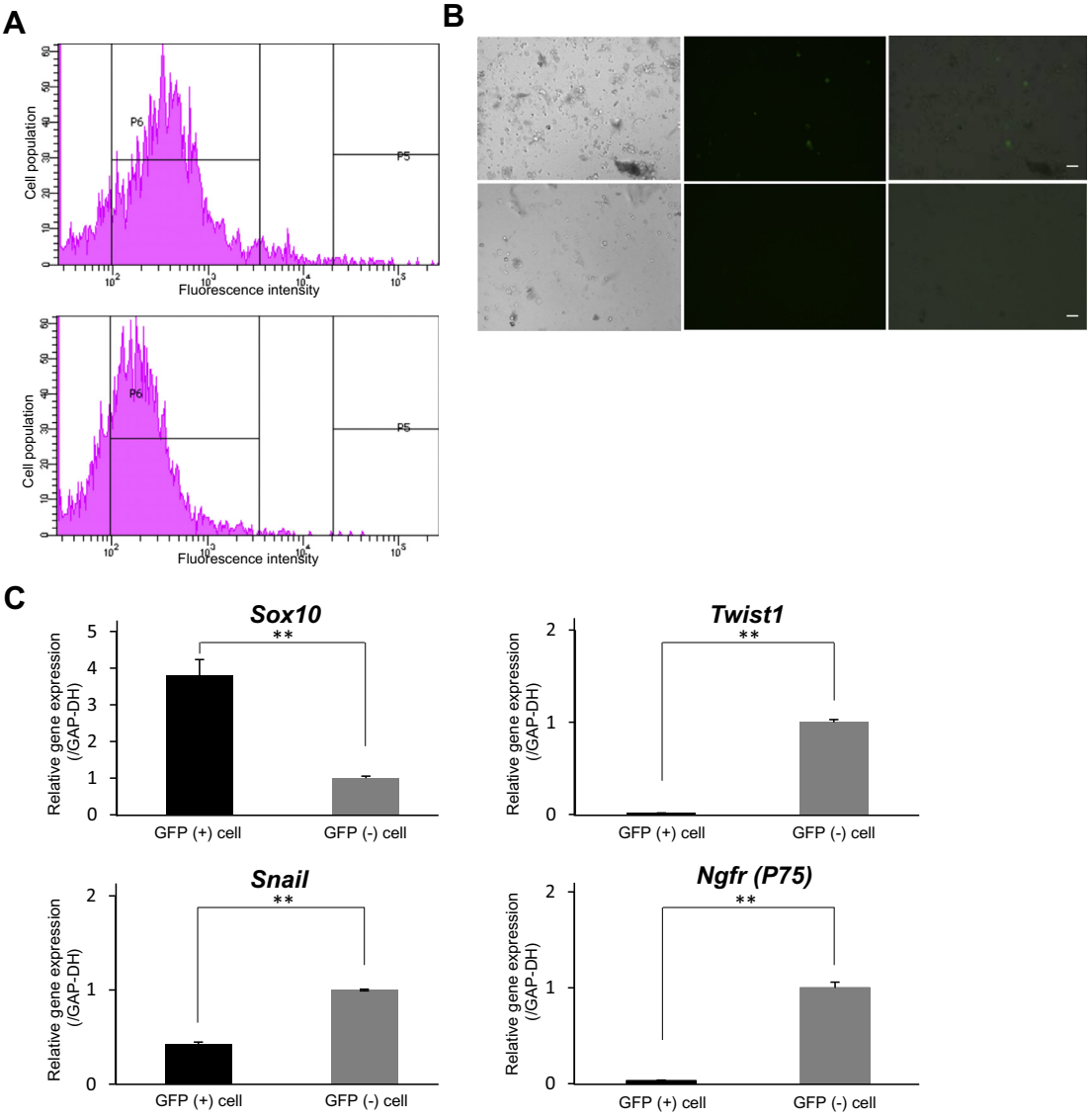

Fig. 2. Isolation of NCDCs from SMGs of adult P0 mice. (A) Expressions of GFP-positive and -negative cells from SMGs of postnatal 8-week-old mice gated by flow cytometric analysis. Top histogram shows flow cytometry data of P0 mice. Bottom histogram shows flow cytometry data of wild-type mice. Gates (higher fluorescence area) in the right-side panels show the GFP-positive cell population and those in the left side (lower fluorescence area) show the GFP-negative cell population. (B) Fluorescence microscope images of GFP-positive (top panel) and -negative (bottom panel) cells isolated by cell sorting (left: phase-contrast image; middle: GFP-fluorescence image; right: merged image). Scale bar = 50 μm. (C) Gene expressions of embryonic neural crest-specific markers in adult SMGs of P0 mice. Real-time PCR analysis revealed that GFP-positive cells have higher expression levels of *Sox10* than GFP-negative cells. GFP(+): GFP-positive cells; GFP(−): GFP-negative cells. Data were normalized with GAPDH as the endogenous control and are shown as the average ± SD of 3 independent experiments ($^{*}p < 0.05$, $^{**}p < 0.01$).

[5,18,19]. GFP-positive cells were found in SMGs of adult P0 mice, which were shown as tiny bright islands in the overall SMG samples, where those were not detected in those of wild-type mice (Fig. 1). Our observations suggest that GFP-positive NCDCs migrate into and are present in adult SMGs. The present results obtained using P0 mice are the first to identify NCDCs in SMGs of adult mice.

3.2. Morphology of SMGs after sorting GFP-positive and -negative cells

To isolate GFP-positive cells from SMGs of adult P0 mice, analysis was performed using flow cytometry. We passed cell suspensions through an FACSAria II to separate and collect GFP-positive and -negative cells. GFP-positive cells were determined based on the intensity of fluorescence. In Fig. 2A, the top histogram shows discrimination of cells obtained from P0 mice, while that on the bottom shows cells from wild-type mice. Cells from the wild type did not show a high level of GFP expression. We also determined the gate of GFP-positive cells, which did not include cells from the wild type. The gates were demarcated to sort GFP-positive and -negative cells, with the former comprising approximately 0.4% of the total cell population in SMGs of adult mice. The number of GFP-positive cells isolated from SMGs of 3 mice ranged from 6×10^4 to 8×10^4.

After separating GFP-positive and -negative cells from SMGs of adult P0 mice by cell sorting, we observed them using fluorescence microscopy (Fig. 2B). We found no contamination of GFP-positive cells in the GFP-negative population, indicating that they could be separated from adult SMGs by cell sorting. Furthermore, we performed real-time PCR analysis to evaluate whether GFP-positive cells in adult SMGs express the embryonic neural crest-related marker genes *Sox10*, *p75*, *snail*, and *twist1* [10,13–16].

GFP-positive cells obtained from adult SMGs expressed a high level of *Sox10* as compared to GFP-negative cells (Fig. 2C). The Sox protein family is essential for many aspects of nervous system development. *Sox10* expression starts in late pre-migratory NCCs and is maintained in most migratory neural crest progenitors, including those that contribute to the peripheral nervous systems [26]. Furthermore, in migrating NCCs, *Sox10* acts as a survival factor and inhibits premature neuronal differentiation [27]. In another study, $Sox10^+/Kit^+$ NCDCs maintained multipotency even after

Table 1
Genes exhibiting higher or lower expression in GFP-positive cells as compared to GFP-negative cells.

Symbol	Name	Expression ratio (GFP+/GFP−)		Feature	Identifier
Gpr4	G protein-coupled receptor 4 (Gpr4)	7.552843	Up	Receptor	NM_175668
Sox15	SRY-box containing gene 15 (Sox15)	6.6790266	Up	Transcription factor	NM_009235
Amy1	Amylase 1, salivary (Amy1)	5.5372434	Up	Enzyme	NM_007446
Aqp5	Aquaporin 5 (Aqp5)	3.881612	Up	Transmembrane channel	NM_009701
Sox10	SRY-box containing gene 10 (Sox10)	2.8716571	Up	Transcription factor	NM_011437
Ednrb	Endothelin receptor type B (Ednrb)	1.0069385	Up	Receptor	NM_007904
Tgfbr2	Transforming growth factor, beta receptor II (Tgfbr2)	3.129647	Down	Receptor	NM_009371
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide (Pdgfra), transcript variant 1	4.220215	Down	Receptor	NM_011058
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide (Pdgfrb), transcript variant 2	8.632226	Down	Receptor	NM_008809

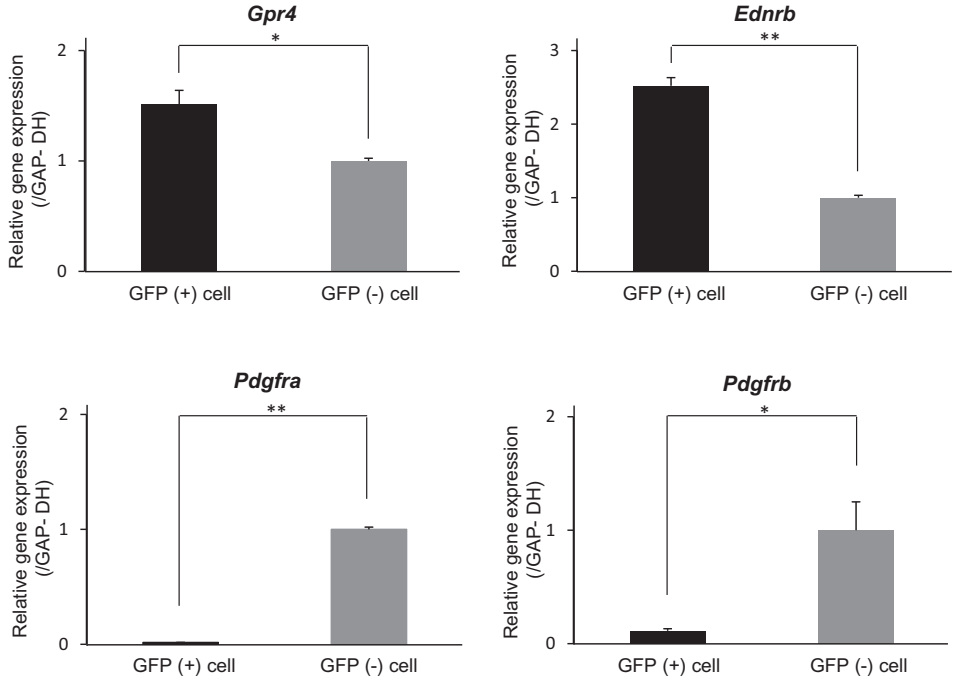

Fig. 3. Analysis of gene expressions for cell surface molecules collected by DNA microarray analysis. As compared to GFP-negative cells, real-time PCR analysis revealed that GFP-positive cells expressed *Gpr4* and *Ednrb* at higher levels, and *Pdgfra* and *Pdgfrb* at lower levels. GFP(+): GFP-positive cells; GFP(−): GFP-negative cells. Data were normalized with GAPDH as the endogenous control and are shown as the average ± SD of 3 independent experiments ($^*p < 0.05$, $^{**}p < 0.01$).

entry into target tissues [28]. Together, these reports indicate that *Sox10* may be a reliable marker for NCDCs during early embryogenesis through adulthood. On the other hand, the expressions of *P75*, *Twist1*, and *Snail* were lower in GFP-positive cells as compared to GFP-negative cells, though the present results did not agree with previous studies [6]. We speculated that this discrepancy in gene expression patterns may be due to tissue-specific characteristics that differ between the adult and embryonic stages.

3.3. Identification of gene expression profile of NCDCs in SMGs of adult mice

Various investigators have reported the precise characteristics of NCCs, including generation, mobilization, and potential for differentiation during embryogenesis. However, appropriate markers for NCDCs in adult mice have not been presented.

To examine whether GFP-positive cells in adult SMGs possess characteristic gene expression profiles, we compared gene expression patterns between GFP-positive cells and -negative cells using DNA microarray analysis, which revealed contrasting patterns (Table 1). As shown in that table, our results indicated that expressions of aquaporin 5 (*Aqp5*) were higher in GFP-positive as compared to GFP-negative cells. Aquaporin is a transmembrane water channel that contributes to membrane water permeability, while *Aqp5* subtype is exclusively expressed in SMGs and responsible for saliva secretion. *Aqp5* has been reported to be acinar cell markers [29]. Hence, there is a possibility that acinar cells have a neural crest origin.

Based on our findings, we then selected genes related to cell differentiation and cell surface molecules, including G protein-coupled receptor 4 (*Gpr4*), which belongs to a protein family comprising 3 closely related G protein-coupled receptors (GPCRs), and functions as a pH sensor and participates in angiogenesis [30,31], endothelin receptor type B (*Ednrb*), a G protein-coupled receptor that activates the phosphatidylinositol-calcium second messenger system [32], and platelet-derived growth factor receptor α (*Pdgfra*) and β (*Pdgfrb*), which have been implicated to control cell proliferation, survival, and migration [33]. Real-time PCR assays were performed to identify and validate novel specific cell surface markers of NCDCs, which in accordance with our microarray data revealed that 4 genes were differentially regulated between GFP-positive and -negative cells (Fig. 3). Those findings, in agreement with microarray results, showed that *Gpr4* and *Ednrb* were up-regulated by 1.5-fold, whereas *Pdgfra* and *Pdgfrb* were down-regulated by 10-fold in GFP-positive as compared to GFP-negative cells. Those two down-regulated genes, *Pdgfra* and *Pdgfrb*, are known to be important for salivary gland development [19].

These results indicate that NCDCs in adult SMGs have characteristic gene expression profiles related to their cell surface molecules. In particular, it is known that *Ednrb* is required for migration of some of NCDCs derivatives, during which it interacts with *Sox10* [34,35]. Furthermore, *Sox10* and the endthelin-3/EDNRB signaling pathway are required for normal enteric nervous system (ENS) and melanocyte development [35]. We found that NCDCs isolated from adult SMGs have high expression levels of *Sox10* and *Ednrb*, which suggest that *Sox10* and the endthelin-3/EDNRB signaling pathway contribute to the morphogenesis of SMGs as well as development of tissues derived from NCDCs, other than the ENS and melanocytes. The present results obtained using P0 mice are the first to show that *Sox10* and EDNRB are related to cell surface markers of NCDCs in SMGs of adult mice.

This is the first study to demonstrate the existence of NCDCs and possibility of isolating them from adult SMGs and our results indicate that some cell surface molecules might be identified as NCDC-specific markers in adult tissues. Cell sorting using a combination of specific cell surface marker genes may be an effective strategy to isolate NCDCs from various adult tissues with high purity. Our results also suggest that these markers may be useful to indicate the transition of NCDCs in some adult tissues.

Acknowledgments

This study was supported by The Project to Establish a Strategic Research Center for Innovative Dentistry by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan, 2010–2014, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan.

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