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Review

Neural Crest-derived Cells in the Oral and Maxillofacial Regions of Adult Mice: Isolation and Application for Regenerative Medicine

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Abstract: Neural crest cells emerge from the dorsal region of the fusing neural tube in vertebrate embryos, then migrate throughout tissues to differentiate into various cell types, 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. Previous studies have suggested that these NCDC subsets persist into adulthood in mammals, especially those cells within the craniofacial compartments. Recently, our group found that NCDCs were scattered throughout tissues of the palate, gingiva, tongue, hair follicle, submandibular glands, and buccal mucosa of adult mice. NCDCs from the buccal mucosa can also form neurosphere-like structures that have the capability to differentiate into osteoblasts in the presence of bone morphogenetic protein-2. In addition, NCDCs in adults have characteristic gene expression profiles, especially their cell surface molecules. Thus, cell sorting using several specific cell surface molecules has been proposed as a useful method for isolating NCDCs with high purity. Together, these results suggest that NCDCs reside in various adult oral and maxillofacial regions, and possess the potential to differentiate into osteoblastic cells, indicating that these cells in adults may be a useful source for bone regeneration strategies. In this review, we discuss the distribution, isolation and osteoblastic differentiation potential of NCDCs isolated from various adult tissue sources in the oral and maxillofacial regions.

Key words: neural crest-derived cells, adult, cellular differentiation, osteoblasts, oral and maxillofacial regions

Introduction

The neural crest (NC) is a transitory structure of the vertebrate embryo that arises from a region at the border of the neural plate, between the neural plate and the adjacent non-neural

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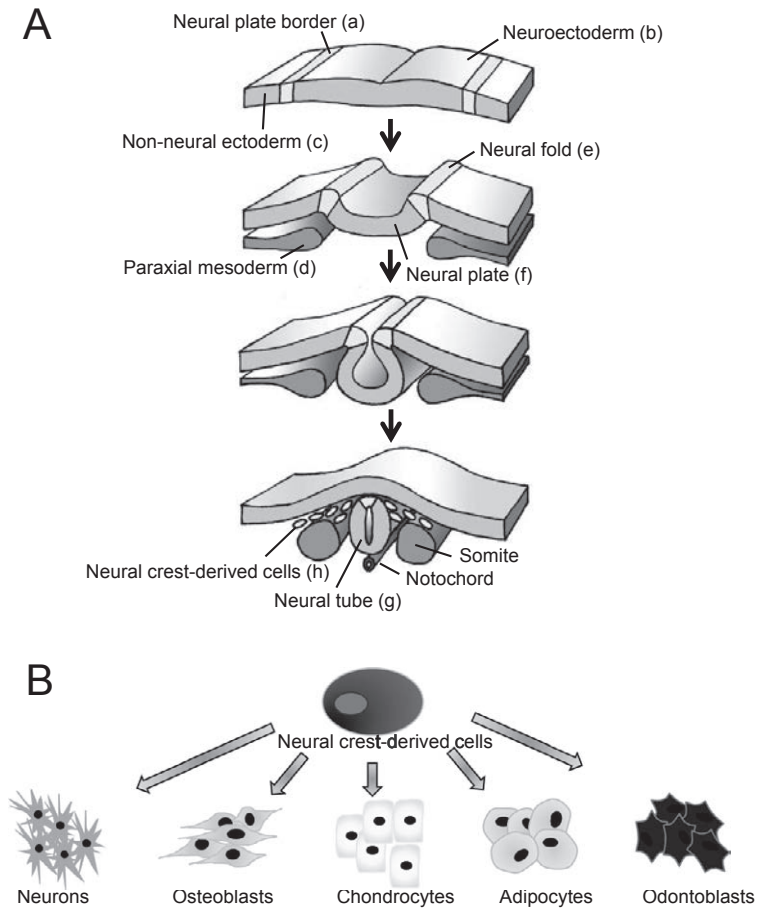

Fig. 1.

(A) The neural plate border (a) is induced by signaling between the neuroectoderm (b) and non-neural ectoderm (c), as well as from the underlying paraxial mesoderm (d). During neurulation, the neural plate borders elevate [neural folds (e)], causing the neural plate (f) to roll into a neural tube (g). Neural crest-derived cells (h) delaminate from the neural folds or dorsal neural tube. *Source:* Adapted from Gammill and Bronner-Fraser (2003)[1] with permission from Nature Publishing Group. Copyright© 2003, Macmillan Publishers Limited. All rights reserved.

(B) Neural crest-derived cell derivatives. Neural crest-derived cells originate from the neural tube and show pluripotency. They migrate extensively, and give rise to a vast array of cell types and organs. In adults, these cells can undergo self-renewal as well as multi-lineage differentiation.

ectoderm. This structure remains at the neural plate border during neurulation and transformation of the neural plate into rising neural folds[1]. After neural tube closure, the NC comes to reside in a domain of the dorsal neural tube (Fig. 1A). Thereafter, NC cells originate from the dorsal aspect of the neural tube[2], undergo an epithelium to mesenchyme transition[3], and become delaminated from the neural tube as NC-derived cells (NCDCs). During vertebrate development, NCDCs migrate in a ventro-lateral manner[4] and populate the branchial arches, while they extensively contribute to the formation of mesenchymal structures in the head during migration[5–7]. Furthermore, NCDCs also have a self-renewal capability and give rise to a multitude of cell types that include neurons, glial cells, myofibroblasts, melanocytes, adipocytes, chondrocytes, osteoblasts, and odontoblasts (Fig. 1B)[8–10].

Recent studies have suggested the continuous survival of these highly pluripotent cells into adulthood, along with their high regenerative potential, and this has been demonstrated in a variety of mammalian craniofacial tissues[11-13]. Interestingly, some NCDCs are maintained in an undifferentiated condition throughout the life of the animal[14, 15], while those obtained from the oral and olfactory mucosa, which are easily accessible in the oral and maxillofacial regions, can be cultured[11-13, 16] and may represent a useful cell source for regeneration of those regions.

In this review, we describe findings regarding the distribution, isolation, and characterization of NCDCs in adult organisms. Moreover, we discuss their specific properties and potential application for cell-based tissue repair strategies.

1. Distribution of neural crest-derived cells in the oral and maxillofacial regions of adult mice

Various investigators have reported the precise characteristics of NCDCs, including generation, mobilization, and differentiation during embryogenesis[8-13]. To analyze the precise distribution and characteristics of NCDCs in adult oral tissues, an established line of double transgenic (*P0-Cre / CAG-CAT-EGFP*) mice (P0 mice) was utilized, in which NCDCs express green fluorescent protein (GFP) throughout the life of the animal[17-20]. *P0*, originally reported as a Schwann cell-specific myelin protein, was previously shown to be transiently activated in migrating NC cells isolated from early chick embryos[21]. *Wnt1* is also expressed specifically in the dorsal neuroepithelium, which includes a pre-migratory population of NC cells in mice[22]. Although *P0* and *Wnt1* are the most reliable markers of NCDCs in embryos, their expressions become completely silenced prior to birth, though recently genetic marking using Cre-recombinase has been applied for long-term tracing of NCDCs in *P0-Cre* and *Wnt1-Cre* mice[8, 17-20]. Using such a technique, we observed the distribution of GFP-positive cells, which were considered to have a NC origin, in the buccal mucosa, palate, gingiva, and tongue samples of adult P0 mice (Fig. 2A, A')[23]. Furthermore, Widera *et al* recently reported the presence of nestin-positive NC-related stem cells within Meissner corpuscles in hard palate tissues of adult rats, and also noted that human palatal cells expressed high levels of the stem cell markers, CD133 and nestin[24]. In agreement with those findings, we observed GFP-positive regions in the palates of adult P0 mice, which also appeared on the surface of the entire palate in a manner similar to the buccal mucosa (Fig. 2B, B'). GFP-positive regions, noted as green dots, were also seen on the surface of the tongue. In addition, Liu *et al* noted contributions of NCDCs to both tongue mesenchyme and epithelium development in 2 independent mouse lines, *Wnt1-Cre* and *P0-Cre*; both express Cre-recombinase in an NC-specific manner[25]. They also reported that NCDCs are distributed within the lingual epithelium and mesenchyme in close association with embryonic taste papillae formation. The GFP-positive cells found in the tongue in that study may be components of taste papillae and taste buds, though additional analysis is necessary to determine the types of cells in the tongue that are positive for GFP[31].

A few studies have noted the existence of NCDCs in embryonic submandibular glands (SMGs)[8, 26]. It is known that embryonic mesenchymal tissues in SMGs are derived from cranial NC cells and we have demonstrated the existence of GFP-positive cells in SMGs of adult P0

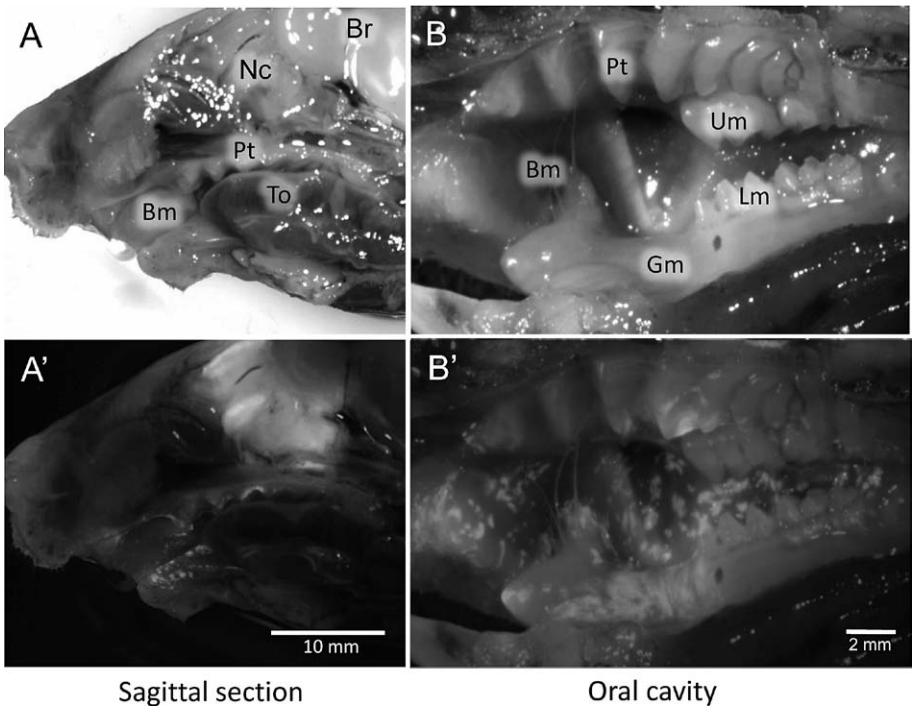

Fig. 2. Distribution of green fluorescent protein-positive cells in adult buccal mucosa, palate, gingiva, and tongue tissues *P0-Cre/CAG-CAT-EGFP* mice. (A) Mid-sagittal section through the skull examined by florescence stereomicroscopy. (B) Representative appearance of sagittal sections obtained from the oral cavity of an adult mouse. Top panels (A, B) show corresponding bright-field images and bottom panels (A', B') show corresponding fluorescence micrograph images. Br, brain; Pt, palate; Bm, buccal mucosa; Gm, gingival mucosa; To, tongue; Um, upper molars; Lm, lower molars. (A, A') Scale bar = 10 mm. (B, B') Scale bar = 2 mm. *Source:* Adapted from Ono *et al* (2015)[23] with permission from Elsevier.

mice, which were shown as tiny bright islands in the overall SMG samples[27]. Another study that utilized *Wnt1-Cre* / ROSA26 transgenic mice showed that embryonic SMG mesenchyme is derived from cranial NCDCs[26], although those NCDCs were restricted to mesenchymal cells in the SMGs and were not found in the epithelium. Platelet-derived growth factor receptor α (*Pdgfra*) and platelet-derived growth factor receptor β (*Pdgfrb*) were also found to be expressed in mesenchymal cells in the SMGs, while GFP-positive cells were observed in the hair follicle bulge, which contains adult stem cells, and the dermal papilla, which retains stem cell-like properties.

2. Clinical potential of adult neural crest-derived cells

The multipotent capacity of NCDCs is a characteristic that indicates their potential for use in regenerative medicine. In general, NCDCs are pluripotent in the early stage of embryonic development, after which their potency becomes more restricted following migration from their niche between the ectoderm and neural tube. However, in recent years, several studies have revealed the persistence of NCDCs in adult mammals. Epidermal NC stem cells transplanted into a contused spinal cord caused improvement in sensory connectivity and substantial recovery of touch

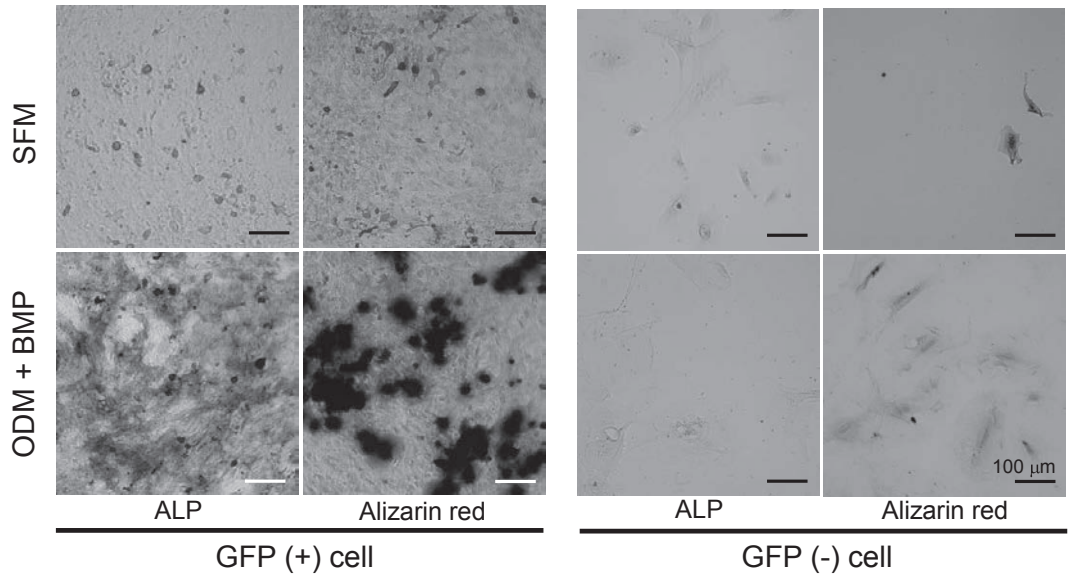

Fig. 3. Osteogenic differentiation of green fluorescent protein (GFP)-positive neural crest-derived cells from the lamina propria of the buccal mucosa of adult *P0-Cre/CAG-CAT-EGFP* mice. Cells were maintained in culture for 14 days after seeding, then subjected to alkaline phosphatase staining (ALP) and alizarin red staining for calcium deposition (alizarin red). GFP-positive [GFP (+)] and -negative [GFP (−)] cells were cultured in osteoblastic differentiation medium (ODM: Minimum Essential Medium with 10% fetal bovine serum containing 10 mM β-glycerol phosphate, 0.5 mM ascorbic acid, and 10^{-8}M dexamethasone) with 200 ng/ml bone morphogenetic protein-2, or sphere-forming medium (SFM: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, supplemented with 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor, B27 supplement). Scale bar = 100 μm. *Source:* Reproduced from Ono *et al* (2015)[23] with permission from Elsevier.

perception[28]. Also, the potential of enteric neural cell therapy for gastrointestinal disorders by transplantation of mouse enteric NC cells into ganglionic and aganglionic mouse gut areas has been revealed *in vivo*. Transplanted enteric NC cells engrafted into ganglionic and aganglionic gut areas showed appropriate spreading and functional integration[29]. Gingivae in the oral and maxillofacial regions were found to contain both NCDCs and mesoderm-derived mesenchymal stem cells (MSCs), with nearly all of the gingival MSCs shown to be derived from cranial NC cells[24]. Also, NC cells derived from gingival MSCs showed an elevated capacity to differentiate into chondrocytes and osteoblasts[30]. In our study, NCDCs were isolated from the lamina propria of the buccal mucosa, and then cultured in sphere-forming and osteoblastic differentiation media. Following isolation of GFP-positive and -negative cells using flow cytometry with a cell sorter, 10%−15% of the buccal mucosa lamina propria cells were found to be positive for GFP[23], When isolated from the lamina propria of the buccal mucosa and cultured, GFP-positive cells formed spheres, a stem cell phenotype, and produced alkaline phosphatase (ALP), a marker enzyme of the early stage of osteoblastic differentiation (Fig. 3). In contrast, GFP-negative cells did not express ALP. Furthermore, mineralization was observed in osteoblastic differentiated GFP-positive cell cultures. Lipid droplets were also noted in adipogenic-stimulated cultures utilizing Oil Red O staining, suggesting that oral NCDCs are multipotent and capable of generating

osteoblasts and adipocytes[23]. Consistent with our results, Davies *et al* suggested that NC-derived progenitors in oral mucosa possess a multipotent capacity[11]. Together, these results suggest that NCDCs in oral mucosa have roles in wound healing.

We also analyzed the potential of NC-derived hair follicle cells to differentiate into osteoblasts in response to bone morphogenetic protein (BMP)-2. Proliferative GFP-positive cells showed increased ALP activity and mineralization in the presence of BMP-2, suggesting that NC-derived hair follicle cells can differentiate into osteoblasts as a result of BMP-2 stimulation. Since abundant GFP-positive regions were seen in the buccal mucosa and hair follicle, we considered that this type of tissue would be beneficial for harvesting NCDCs for use in regenerative medicine strategies.

3. Gene expression profile of neural crest-derived cells in adults

NCDCs have been isolated from multiple sites within the body. Stemple and Anderson identified and isolated NCDCs from the trunk neural tube of rat embryos based on the expression of the low-affinity nerve growth factor receptor (p75)[31]. Thereafter, NCDCs were isolated from the lamina propria of the oral mucosa by differential adhesion to fibronectin[11]. In addition, a connexin-43-enriched human cell population isolated from periodontal ligament cells obtained from extracted third molars was shown to be multipotent in *in vivo* teratoma formation assays[32]. Hauser *et al* recently reported a separation strategy based on magnetic cell sorting of p75-positive inferior turbinate cells, which formed larger neurospheres and proliferated faster than p75-negative inferior turbinate cells[33]. Rat NCDCs were also isolated from the gut on embryonic day 14.5 by selecting p75 and $\alpha 4$ integrin double-positive fractions[34]. Although p75 is useful as a marker for isolation of NCDCs, it is also expressed on other types of cells, and genetic lineage labeling techniques, such as *P0-Cre* and *Wnt1-Cre / EGFP,* are not available for use with human samples. Therefore, it is very important to determine reliable cell surface molecules that are exclusively expressed on NCDCs found in adult human tissues. To examine whether GFP-positive cells in adult SMGs possess characteristic gene expression profiles, we compared gene expression patterns between GFP-positive and -negative cells using DNA microarray analysis, then selected genes related to cell surface molecules based on those findings[24]. Compared to GFP-negative cells, GFP-positive cells expressed G protein-coupled receptor 4 and endothelin receptor type B (*Ednrb*) at higher levels, whereas *Pdgfra* and *Pdgfrb* were expressed at lower levels[27]. Additionally, these two down-regulated genes (*Pdgfra* and *Pdgfrb*) are known to be important for salivary gland development[26].

Together, 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 NCDC derivatives, during which it interacts with *Sox10*, while *Sox10* and the endothelin-3 / EDNRB signaling pathway are required for normal enteric nervous system and melanocyte development[35, 36]. We found that NCDCs isolated from adult SMGs had high expression levels of *Sox10* and *Ednrb*, indicating that *Sox10* and the endothelin-3 / EDNRB signaling pathway contribute to the morphogenesis of SMGs, as well as the development of

tissues derived from NCDCs other than those of the enteric nervous system and melanocytes. This study demonstrated the existence of NCDCs and a possible method for their isolation from adult SMGs, and our results suggest that some cell surface molecules might be identifiable as NCDC-specific markers in adult tissues.

In adults, NCDCs widely reside throughout the oral and maxillofacial regions, such as the palate, gingiva, tongue, and buccal mucosa, suggesting an important role. These tissues are also easily accessible for resection with a minimally invasive surgical procedure. NCDCs isolated from adult oral tissues have a stem cell phenotype, while they have also been shown to proliferate and differentiate into osteoblastic cells *in vitro*. Furthermore, cell sorting using a combination of specific cell surface marker genes may be an effective strategy to isolate NCDCs from various adult tissues with a high level of purity. We propose that NCDCs in oral tissues may be an ideal candidate cell source for regenerative medicine strategies.

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Conflict of interest disclosure

The authors have no conflict of interest to declare.

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