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# Generation, Differentiation, and Maturation of Olfactory Receptor Neurons In Vitro

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**Key words.** Olfactory epithelium—Neurogenesis—Neuronal precursor—Polypeptide growth factor—Cell migration—Extracellular matrix

## Introduction

In the mammalian olfactory epithelium (OE), proliferation of neuronal precursor cells and differentiation of their progeny into olfactory receptor neurons (ORNs) are ongoing processes [1]. The OE is thus an ideal system for exploring how neurogenesis can control neuron number. There is also evidence that cell interactions regulate neurogenesis in the OE: Degeneration of ORNs following removal of the olfactory bulb (their central target) or lesioning of ORN axons leads to increased proliferation of neuronal precursor cells in the epithelium and subsequent production of new receptor neurons [2].

Although new ORNs are clearly generated in bulbectomized animals, the full complement of biochemically mature neurons is never reconstituted [3]. That immature ORNs are generated at all in bulbectomized animals suggests that neurogenesis and neuronal differentiation occur independently of target tissue [4]. However, these *in vivo* experiments also suggest that subsequent biochemical maturation and survival of ORNs are to some extent target tissue-dependent [3].

We have developed a culture system for OE purified from mouse embryos [4]. During our initial characterization of OE explants, we identified three cell types: (1) basal cells, which grow as epithelial sheets and express keratins; (2) postmitotic olfactory receptor neurons (ORNs), which express the neural cell adhesion molecule N-CAM; and (3) immediate neuronal precursors (INPs), which do not express keratins or N-CAM. INPs are migratory round cells that rapidly sort out from basal cells, synthesizing DNA and dividing as they migrate. <sup>3</sup>H-Thymidine

(<sup>3</sup>H-TdR) incorporation analysis indicates that INPs are the direct precursors of ORNs [4].

Interestingly, the events that occur during bulbectomy-induced neuronal degeneration and regeneration *in vivo* in the OE appear to be mimicked *in vitro* in OE cultures: Proliferation of INPs and differentiation of ORNs occur in the absence of target tissue [4]. However, genesis of ORNs from INPs continues for only 1 to 2 days and then ceases. Cessation of neurogenesis *in vitro* may reflect inappropriate or inadequate mitogenic stimulation of INPs, as studies suggest that INPs may be able to undergo as many as three rounds of division *in vivo* [5]. With time in culture, not only do the INPs cease dividing and disappear (owing to their generation of ORNs), but ORNs begin to die and by 7 days are no longer detectable [4]. *In vivo*, contact with the olfactory bulb appears to enable ORNs to survive for prolonged periods [6]. Because no central nervous system (CNS) cells are present in our OE cultures, any trophic factors or cell interactions normally supplied by the CNS are necessarily absent.

Our previous studies in this system suggest that olfactory neurogenesis and neuron survival *in vitro* are subject to regulatory influences such as those that operate *in vivo*, in that extrinsic agents are clearly required to permit long-term neuronal production and survival. Here we describe results from experiments designed to identify and characterize molecules that regulate generation, differentiation, and maturation of ORNs. Our experiments focus on two classes of molecules: polypeptide growth factors and glycoproteins of the extracellular matrix (ECM).

## Materials and Methods

### Materials

Recombinant human nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and recombinant rat ciliary neurotrophic factor (CNTF) were obtained from Genentech (San Francisco, CA). Recombinant human acidic fibroblast growth factor (FGF1), basic fibroblast growth factor (FGF2), transforming

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growth factor- $\alpha$  (TGF- $\alpha$ ), epidermal growth factor (EGF), and platelet-derived growth factors AA and BB (PDGF-AA, PDGF-BB) were obtained from US Biochemicals (Cleveland, OH). Recombinant human transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) was from Genzyme (Cambridge, MA). Laminin, fibronectin, merosin, and poly-D-lysine (PDL) were prepared or purchased as described [7]. For explant cultures, acid-cleaned glass coverslips were prepared and coated with ECM molecules as described [4,7]. For multiwell cultures, 96-well tissue culture trays (Costar no. 3596) were coated overnight with PDL (1 mg/ml in water), washed, and sterilized by ultraviolet (UV) light. AG1D5 monoclonal anti-N-CAM antibody was prepared as described [4]. Anti-MASH1 hybridoma 24B72D11 was the gift of D. Anderson (California Institute of Technology). Monoclonal anti-Thy 1.1 (Ab22) was the gift of W. Matthew (Duke University Medical School).

### Tissue Culture and Analysis

For assessing growth factor effects on neuronal precursor proliferation, explants of purified OE from embryonic day 14–15 (E14–15) CD-1 mice were cultured on glass coverslips coated with PDL followed by merosin (10  $\mu$ g/ml) for 3 h at 37°C [4,7]. Cultures were grown in serum-free, low calcium (0.1 mM) medium with modified N2 additives (LCM) [4], except that bovine serum albumin (BSA; Clinical Reagent Grade, ICN) was reduced to 1 mg/ml. Cultures were grown for 24 h in the tested growth factor, then growth factor was replenished, and  $^3$ H-TdR (0.1  $\mu$ Ci/ml; 80 Ci/mmol) was added. After another 24 h, cultures were fixed and processed for autoradiography [4]. Quantitative analysis of  $^3$ H-TdR incorporation was performed using explants as statistical units. For each explant analyzed, the number of migratory cells incorporating  $^3$ H-TdR was counted and this number normalized to the explant area. This labeling index was measured for a minimum of 20 to 40 explants per experimental condition. The proliferation factor (PF) was then calculated as a ratio: mean labeling index for that condition/mean labeling index for explants grown in no growth factor. Percent error of the ratio was calculated as the square root of the sum of the squares of percent errors (from SEMs) of the two labeling indices being compared; it was generally around 20%.

For determining adhesion and migratory responses of olfactory neuronal cells, either E14–15 explants, or the "neuronal cell" fraction (ORNs plus INPs) of OE suspension cultures (prepared as described [7]) were cultured in LCM containing crystalline BSA 5 mg/ml [7]. For assessing effects of growth factors and pharmacologic agents on survival and maturation of ORNs, the neuronal cell fraction of OE suspension cultures was also used, except that

OE was purified from E16–17 homozygous OT-2 transgenic mice [8], and neuronal cell fractions were prepared after 6 h of suspension culture without the use of proteolytic enzymes. Neuronal cells were plated onto PDL-coated 96-well tissue culture trays at  $2 \times 10^4$  to  $5 \times 10^4$  cells per well in LCM with crystalline BSA 5 mg/ml.

## Results and Discussion

### Mitogenic Effects of Polypeptide Growth Factors on Olfactory Neuron Precursors

Compelling evidence suggests that two families of polypeptide growth factors—neurotrophins (NTs) and fibroblast growth factors (FGFs)—are important regulators of neuron number: Both families have widespread expression in the nervous system [9,10], both exert trophic effects *in vivo* and *in vitro* on a wide variety of neurons [11], and there is evidence for their mitogenic effects on neuronal precursors as well [12].

Receptors for NTs and FGFs appear to be expressed by OE cells *in vivo*: Affinity cross-linking experiments have demonstrated cross-linking of both BDNF and NT3 to membranes from E15 olfactory turbinates (E. Escandon, K. Nikolic, A. Calof, unpublished data). Reverse transcriptase-polymerase chain reaction analysis of RNA from E15 OE indicates that FGF receptors FR1 and 2 are also expressed [13]. To determine whether NTs, FGFs, or other families of polypeptide growth factors affect proliferation of olfactory neuron precursors, we measured the relative proliferation of migratory olfactory neuronal cells grown for 48 hours in the presence of various factors. Proliferation was assessed by measuring  $^3$ H-TdR incorporation from 24 to 48 h in culture, a time when neurogenesis has greatly diminished in OE cultures grown in defined, serum-free medium [4]. Growth factors tested were FGF1 (100 ng/ml), FGF2 (10 ng/ml), PDGF $\alpha\alpha$  (10 ng/ml), PDGF $\beta\beta$  (10 ng/ml), EGF (20 ng/ml), TGF- $\alpha$  (20 ng/ml), BDNF (50 ng/ml), NT3 (50 ng/ml), NGF (50 ng/ml), CNTF (10 ng/ml), and TGF- $\beta$ 2 (10 ng/ml). Only FGF1 and FGF2 caused a statistically significant increase in the proliferation of olfactory neuronal cells. FGF2 showed the most pronounced effect, giving a three- to fourfold increase in proliferation ( $\overline{PF} = 3.23$ ); the effect of FGF1 was slightly less ( $\overline{PF} = 2.82$ ) [13].

To verify that labeled migratory cells in these assays were ORN precursors, it was demonstrated that 85% to 90% of  $^3$ H-TdR-labeled cells expressed immunologically detectable N-CAM (a marker for ORNs) within 24 to 36 h following a brief (2-h) pulse of  $^3$ H-TdR (5  $\mu$ Ci/ml; 80 Ci/mmol). This proportion was similar for cultures pulsed at  $t = 10$  and  $t = 22$  h in culture and for cultures grown in the presence or absence of FGF2. Thus most proliferating migratory

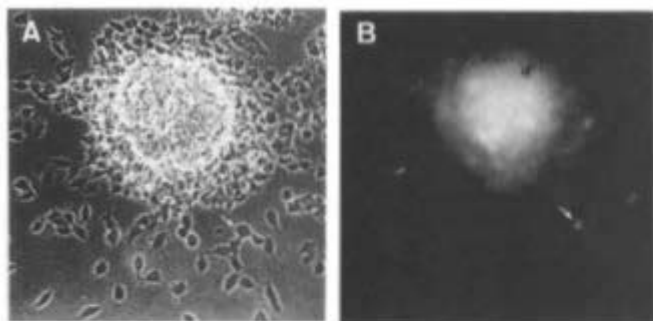


FIG. 1. Anti-MASH1 immunostaining of olfactory epithelium cultures. E14 OE explants were grown for 10 h, then fixed and stained with anti-MASH1 hybridoma supernatant followed by rhodamine-conjugated goat anti-mouse IgG (Zymed, 1:30). **A** Phase contrast. **B** Rhodamine optics. Arrow points to a MASH1<sup>+</sup> nucleus

cells in these cultures are INPs, and FGF2 apparently acts on this population [13].

#### Immortalization and Immunologic Analysis of Cultured Olfactory Epithelium Cells: New Classes of Precursor Cells

Although INPs may be identified by their morphology, lack of keratin and N-CAM immunoreactivity, and ability to migrate and incorporate <sup>3</sup>H-TdR, they may consist of more than one kind of precursor cell. We have found that a monoclonal antibody to the transcription factor MASH1 [14], a homolog of *Drosophila achaete-scute* proteins, recognizes a subset of migratory cells in early ( $t = 10$  h) explant cultures of E14–15 OE (Fig. 1). MASH1-immunoreactive cells appear to be dividing, as they incorporate <sup>3</sup>H-TdR (not shown), but only a small proportion (3%–4%) of total migratory, <sup>3</sup>H-TdR-incorporating cells are MASH1<sup>+</sup>. This finding suggests that MASH1<sup>+</sup> cells are a subset of INPs, perhaps at a specific stage of differentiation. The possibility that MASH1<sup>+</sup> cells in OE cultures are in fact progenitors of INPs is suggested by evidence that targeted disruption of the mouse MASH1 gene causes loss of most of the ORNs (F. Guillemot, personal communication).

Generation of immortalized cell lines from proliferating cells in OE cultures has revealed that progenitors for olfactory ensheathing cells are present in cultures of OE purified from E14–15 mice [15]. These cell lines express markers characteristic of olfactory ensheathing cells, including glial fibrillary acidic protein (GFAP) and S-100 [16], but no growth condition we have yet tried has caused them to express markers of neuronal differentiation, suggesting that ensheathing cells are in a lineage separate from that of ORNs.

#### Effects of Extracellular Matrix Molecules on Cell Migration and Axon Outgrowth by Olfactory Neuronal Cells

In vivo, neurons derived from OE exhibit two types of motile behavior. Differentiating receptor neurons

extend axons that grow through the olfactory nerve to targets in the olfactory bulb; and in the embryo some cells also leave the epithelium entirely and migrate into the brain, where they become luteinizing hormone releasing hormone-secreting neurons of the hypothalamus [17]. In vitro, neurons and INPs of the OE exhibit both types of behavior [4,7]. Interestingly, cell migration and neurite outgrowth occur on substrata treated with the ECM protein laminin or its homolog merosin but not on substrata treated with other ECM molecules, such as fibronectin or collagens [7]. The effects of laminin can be mimicked by an elastase fragment of laminin known as E8 and can be blocked by antibodies to integrin receptor  $\alpha 6 \beta 1$ , which is known to be a receptor for E8 [18]. Purified OE neuronal cells are also stimulated to migrate and extend neurites by laminin, but laminin is not adhesive for these cells [7]. In contrast, fibronectin supports strong neuronal adhesion but does not promote cell migration [7]. These results suggest that laminin, working through an integrin receptor, stimulates olfactory neuron motility via a mechanism other than promotion of cell-substratum adhesion.

#### Regulation of Olfactory Receptor Neuron Maturation and Survival

Using OT-2 transgenic mice, we are able to identify—and potentially purify—OE cells that can be unambiguously identified as mature ORNs (MORNs). In the OT-2 strain, regulatory elements of the olfactory marker protein gene drive expression of the Thy 1.1 cDNA in mice that are otherwise homozygous for Thy 1.2. Therefore MORNs are the only cells in OT-2 mice that express Thy 1.1 [8].

Initial experiments have examined effects of NTs and pharmacologic agents on cultures of ORNs prepared from E16–17 OT-2 embryos. We have found that aurintricarboxylic acid (AT; 100  $\mu$ M), which prevents programmed cell death in other neuronal cells in vitro [19], promotes survival of cultured ORNs as well. NTs can also promote survival of ORNs in vitro, with AT > BDNF > NT3 > NGF in effectiveness (not shown). Interestingly, the number

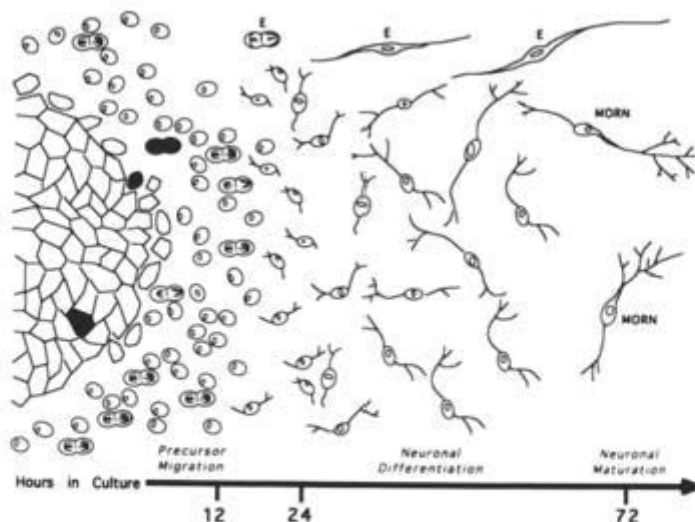


FIG. 2. Timeline of cellular differentiation in olfactory epithelium cultures. Cellular transitions occurring in E14–E15 OE explant cultures are shown, from left to right, over the time course in which they occur. Shortly after explants are plated, olfactory neuron precursors (primarily INPs) sort out from basal epithelial cells and migrate extensively when plated on laminin-treated substrata. A

subpopulation of these precursors (shown in *black*) are *MASH1*<sup>+</sup>. Over the course of 2 days in culture, progeny of INPs differentiate into ORNs. When cultured under conditions that inhibit programmed cell death, ORNs mature biochemically (*MORN*, mature ORN), even in the absence of target tissue. *E*, progenitors of olfactory ensheathing cells, also derived from epithelial cells in OE cultures

of Thy 1.1-expressing ORNs surviving for 72 h in AT appears to be greater than the number that were present at the time of initial plating [20]. This suggests that AT treatment allows biochemical maturation of ORNs in the absence of their target tissue, suggesting that both initial differentiation [4] and subsequent biochemical maturation of ORNs are target-independent developmental processes.

## Summary

Tissue cultures of embryonic mouse OE have been used to functionally test molecules regulating generation, differentiation, and maturation of ORNs. These *in vitro* experiments have also led to new insights into the cellular stages of olfactory neurogenesis. Our current concept of this process as it occurs in OE cultures is pictured in Fig. 2.

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