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Progenitor Cells of the Olfactory Receptor Neuron Lineage

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KEY WORDS stem cell; neurogenesis; transcription factors; bone morphogenetic proteins; fibroblast growth factors

ABSTRACT The olfactory epithelium of the mouse has many properties that make it an ideal system for studying the molecular regulation of neurogenesis. We have used a combination of in vitro and in vivo approaches to identify three distinct stages of neuronal progenitors in the olfactory receptor neuron lineage. The neuronal stem cell, which is ultimately responsible for continual neuron renewal in this system, gives rise to a transit amplifying progenitor identified by its expression of a transcription factor, MASH1. The MASH1-expressing progenitor gives rise to a second transit amplifying progenitor, the Immediate Neuronal Precursor, which is distinct from the stem cell and MASH1-expressing progenitor, and gives rise quantitatively to olfactory receptor neurons. Regulation of progenitor cell proliferation and differentiation occurs at each of these three cell stages, and growth factors of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families appear to play particularly important roles in these processes. Analyses of the actions of FGFs and BMPs reveal that negative signaling plays at least as important a role as positive signaling in the regulation of olfactory neurogenesis. *Microsc. Res. Tech.* 58:176-188, 2002. © 2002 Wiley-Liss, Inc.

INTRODUCTION

To study basic questions concerning the regulation of progenitor cell proliferation and differentiation during development and regeneration of the mammalian nervous system, our laboratory has concentrated on the olfactory epithelium (OE) of the mouse as a model experimental system. The OE of a mature mouse can be considered of consist of three main cell compartments, which are illustrated in Figure 1. The apical compartment, adjacent to the nasal cavity, contains a single layer of supporting (or sustentacular) cells. Olfactory receptor neurons (ORNs), the odor-transducing cells of the OE, are found in the middle, largest compartment of the epithelium. Within this middle compartment, several layers of mature ORNs, which express olfactory marker protein (OMP) and have established connections with the olfactory bulb, are located above several layers of immature ORNs, which express the growth-associated protein GAP43 (Verhaagen et al., 1989). Both immature and mature ORNs express the neural cell adhesion molecule, NCAM (Calof and Chikaraishi, 1989). Basal cells of the OE consist of two broad morphological subtypes: horizontal basal cells (HBCs) and globose basal cells (GBCs). HBCs lie adjacent to the basal lamina and express keratin intermediate filaments (Calof and Chikaraishi, 1989); these cells do not appear to be part of the neuronal lineage of the OE. The GBCs lie above horizontal basal cells, and it is this population of cells that has been shown to contain the progenitor cells that divide and give rise to ORNs in vivo (Caggiano et al., 1994; Gordon et al., 1995; Mackay-Sim and Kittel, 1991; Schwartz Levey et al., 1991). The OE rests on a basal lamina, beneath which lies a mesenchymal stroma.

Several features of mouse OE make it ideal for studies concerning the molecules and cell interactions that regulate neurogenesis. Most significantly, the OE re-

tains, throughout life, the ability to generate ORNs (Graziadei and Monti Graziadei, 1978). Structurally, the OE is a neuroepithelium similar to the germinative neuroepithelia of the embryo that give rise to the central nervous system (Cuschieri and Bannister, 1975; Smart, 1971). However, the OE is much simpler in that it produces only one major type of neuron, the ORN. Thus, because studies in vitro and in vivo have shown that progenitor cells in the OE behave in a predictable and relatively uniform manner (e.g. Calof and Chikaraishi, 1989; Gordon et al., 1995; Schwartz Levey et al., 1991), it is possible to study the detailed biology of neurogenesis in the OE without the confounding issue of morphological and functional diversity in the neuronal population that arises from progenitor cell proliferation. Moreover, the increasing use of transgenic mouse technology in studying OE neurogenesis is making possible a detailed understanding of the regulation of neurogenesis at the molecular genetic level (e.g., Calof et al., 1996b; Cau et al., 2000; Guillemot et al., 1993; Murray et al., 1998). Ultimately, identifying the molecules and cell interactions that permit ongoing neurogenesis and neuronal regeneration in the OE should provide us with knowledge important for fostering this process in other regions of the nervous system,

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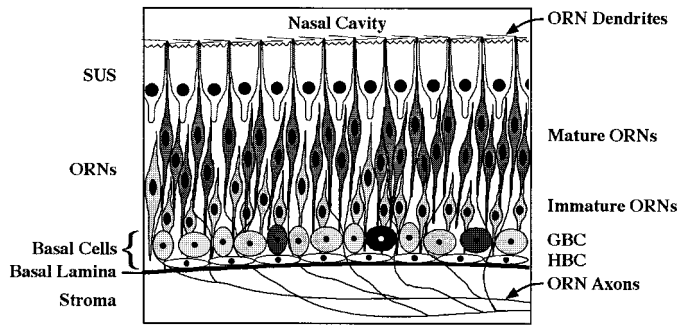


Fig. 1. Cell types of the olfactory epithelium. Cartoon of a section through the OE of an adult mouse, illustrating the positions of the different cell types in the three main cell compartments. From apical to basal, these are sustentacular (supporting) cells (SUS), olfactory receptor neurons (ORNs), and basal cells. There are two broad morphological classes of basal cells: horizontal basal cells (HBC), which are keratin-expressing cells lying adjacent to the basal lamina (Calof and Chikaraishi, 1989) and do not participate in the neuronal lineage (e.g., Caggiano et al., 1994); and globose basal cells (GBC), which consist of the 3 known types of neuronal progenitors: neuronal stem cells (black; thought to be the neuronal colony-forming cells observed in *in vitro* assays; see Mumm et al., 1996); MASH1-expressing progenitors (dark gray), and Immediate Neuronal Precursors (INPs; light gray). The OE is separated from its underlying stroma by a basal lamina.

where it fails to occur in adults (Murray and Calof, 1999).

We have taken advantage of these properties of mouse OE to establish simple and reliable techniques for generating OE cultures in which neurogenesis occurs efficiently and can be studied quantitatively, as well as to identify molecular markers for major cell types in the OE (reviewed in Calof et al., 1999). We use three main tissue culture assays for our studies: (1) OE explant cultures, which are initiated as sheets of intact OE, purified from underlying stroma during tissue preparation. In OE explants, cells of the neuronal lineage migrate away from explanted pieces of OE, proliferating as they migrate; such cultures are particularly useful for studies of neurogenesis because they allow quantification of migratory cells and use of cell-type-specific markers in conjunction with markers of cell proliferation (e.g., BrdU; see DeHamer et al., 1994). (2) We have also developed cultures consisting of a dissociated "neuronal cell fraction" isolated from OE, which contains solely ORNs and their progenitors. This culture assay is useful for examination of growth factor effects on proliferation and survival of ORNs and their progenitors (e.g., DeHamer et al., 1994; Holcomb et al., 1995). (3) The neuronal colony-forming assay, which consists of OE neuronal progenitor cells purified by immunological panning from the dissociated neuronal cell fraction, plated on feeder layers of mitotically-inactivated OE stromal cells (Mumm et al., 1996). Neuronal colony-forming assays have been important in providing information about putative OE neuronal stem cells, and have also proved to be extremely useful for identifying signaling molecules that have both positive and negative effects on OE neurogenesis (Mumm et al., 1996; Shou et al., 1999b, 2000).

Through the use of these tissue culture assays in combination with *in vivo* assays of induced neurogenesis, we have identified major cell stages in the ORN

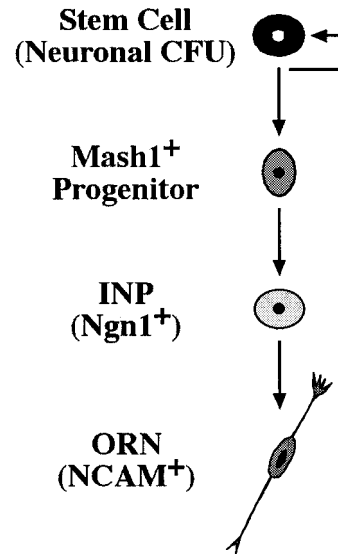


Fig. 2. A proposed model of the neuronal lineage in the olfactory epithelium. Our studies have identified three stages of neuronal progenitor cells in the ORN lineage: mammalian achaete-scute homologue-1 (MASH1) expression demarcates cells at the earliest transit amplifying stage; this stage appears to be interposed between the neuronal stem cell (tentatively identified as a neuronal colony-forming cell in tissue culture assays), and the Immediate Neuronal Precursor (INP), which expresses *Ngn1*. INPs are the last proliferating cells in the ORN lineage: they undergo one or more rounds of division, after which their progeny differentiate into NCAM-expressing ORNs. ORNs are postmitotic cells. For detailed description, see text.

lineage and have described their precursor-progeny relationships. Our current concept of the ORN lineage is illustrated in Figure 2. Summarized briefly, the neuronal stem cell of the OE has been tentatively identified as the cell that gives rise to neuronal colonies in neuronal colony-forming assays (i.e., the neuronal colony-forming cell or neuronal CFU; see Mumm et al., 1996). The progeny of the neuronal stem cell is thought to be the MASH1-expressing neuronal progenitor, which undergoes 1–2 rounds of division to give rise to Immediate Neuronal Precursors (INPs; Gordon et al., 1995). INPs divide 1–2 times *in vitro* as well (cell cycle length \approx 17 hours), and then quantitatively give rise to postmitotic ORNs, which express the neural cell adhesion molecule within a few hours of their terminal S-phase (Calof and Chikaraishi, 1989; DeHamer et al., 1994). Below we describe in detail our current knowledge of the identity and function of these three types of neuronal progenitors.

PROGENITOR CELLS OF THE OLFACTORY RECEPTOR NEURON LINEAGE Neuronal Stem Cell

The OE has long been thought to harbor a neuronal stem cell because generation of ORNs takes place continually in this tissue, from fetal through adult life (Graziadei and Monti Graziadei, 1978). Studies of neurogenesis and induced neuronal regeneration in adult animals indicate that the neuronal stem cell resides in the basal compartment of the OE (Camara and Harding, 1984; Gordon et al., 1995; Graziadei, 1973; Hinds et al., 1984; Mackay-Sim and Kittel, 1991; Moulton and

TABLE 1. Cell types of the olfactory receptor neuron lineage¹

Cell stage in vitro	References	Cell type in vivo	Molecular marker in vivo	References
Neuronal colony forming cell	(Mumm et al., 1996)	GBC	Hes1* BF-1*	(Cau et al., 2000) (Hatini et al., 1999; Yao et al., 2001)
Mash1+ progenitor	(Gordon et al., 1995)	GBC	Mash1 OAZ* BF-1* Otx-2* Hes1*	(Cau et al., 1997; Gordon et al., 1995) (Tsai and Reed, 1997) (Hatini et al., 1999); Fig. 3B (this paper) (Calof et al., 1996a; Simeone et al., 1993) (Cau et al., 2000)
INP	(Calof and Chikaraishi, 1989)	GBC	Ngn1 OAZ* BF-1* Otx-2* Hes1*, Hes5*	(Cau et al., 1997); Fig. 3A (this paper) (Tsai and Reed, 1997) (Hatini et al., 1999); Fig. 3b (this paper) (Calof et al., 1996a; Simeone et al., 1993) (Cau et al., 2000)
ORN	(Calof and Chikaraishi, 1989)	ORN	NCAM, GAP43 OMP NeuroD Class III β-tubulin, SCG10 Olf-1 Peripherin Stathmin	(Calof and Chikaraishi, 1989; Verhaagen et al., 1989) (Farbman and Margolis, 1980); Fig. 14 (this paper) (Cau et al., 1997) (Guillemot et al., 1993) (Wang and Reed, 1993) (Gorham et al., 1991) (Camoletto et al., 2001)

¹Cell stages identified in tissue culture assays of OE neurogenesis are listed along with their in vivo counterparts, as well as molecular markers for each cell type. Markers indicated with an asterisk (*) have not yet been confirmed.

Fink, 1972; Schwartz Levey et al., 1991). Retroviral lineage analyses have confirmed that all neuronal progenitors of the ORN lineage (including, presumably, stem cells) reside in basal OE (Caggiano et al., 1994). Such studies have also contributed to the realization, derived initially from developmental and immunohistochemical experiments, that neither horizontal basal cells nor sustentacular cells are within the ORN lineage (Klein and Graziadei, 1983; Matulionis, 1976; Mulvaney and Heist, 1971).

To date, despite much effort, no specific molecular marker of the neuronal stem cell of the OE has been identified. The earliest progenitor cell type that can be definitively identified in the ORN lineage is the MASH1-expressing progenitor, which is a proliferating cell type early in the lineage, but which does not appear to be a self-renewing stem cell (see below and Gordon et al., 1995). MASH1 is a basic-helix-loop-helix transcription factor that was cloned based on its homology to proneural genes of the *achaete-scute* complex in *Drosophila* (Johnson et al., 1990). In *Drosophila*, expression of proneural genes is regulated by the prepattern genes, which include the genes *hairy* and *enhancer of split* (Skeath and Carroll, 1991, 1994). A mammalian homologue of these prepattern genes, *Hes1*, is expressed in the olfactory placode, the primordium of the OE, and appears to function upstream of *Mash1* (Cau et al., 2000). Thus, although this idea has yet to be tested, *Hes1* is likely to be expressed by the neuronal stem cell of the OE. However, since *Hes1* is also expressed by progenitor cells downstream of the MASH1-expressing progenitor in the OE (see below and Table 1), it may not be useful as a specific molecular marker of the neuronal stem cell (Cau et al., 2000).

Another potential marker for the neuronal stem cell is the winged-helix transcription factor, brain factor 1 (*BF-1*, Tao and Lai, 1992; also known as *Foxg1*, Kaestner et al., 2000). *BF-1* is expressed early in the developing olfactory placode (Tao and Lai, 1992), and

in *BF-1* null embryos, the OE is greatly reduced in size and most proliferating cells are absent (Hatini et al., 1999). Such findings suggest that *BF-1* is expressed by an early progenitor in the ORN lineage. However, as shown in Figure 3, we have examined expression of *BF-1* in the OE of e14.5 mouse embryos, and the pattern of *BF-1* expression encompasses more cells than are expected to be neuronal stem cells, based on calculations of stem cell number in the OE at this age (Murray and Calof, unpublished observations; see DeHamer et al., 1994; Mumm et al., 1996). Thus, as is the case for *Hes1*, *BF-1* expression may not be restricted to neuronal stem cells (Table 1). Ultimately, studies of gene expression during neuronal regeneration in adult animals, as well as neurogenesis in embryos, will help to confirm the molecular identity of the OE neuronal stem cell.

Is there evidence for the existence of OE neuronal stem cells in vitro? Our initial clue that OE stem cells are present and can function in vitro came from studies of the actions of fibroblast growth factors (FGFs) on OE explant cultures (DeHamer et al., 1994). These studies showed that treatment with FGFs causes a small subpopulation of explants (about 5% of all explants) to continue to generate large numbers of both proliferating neuronal progenitors and differentiated ORNs for 4–5 days in culture. This is illustrated in Figure 4. Data such as these suggest the presence, in rare OE explants, of neuronal stem cells that depend upon FGFs for their ability to survive and/or proliferate (DeHamer et al., 1994). Interestingly, one of the FGFs that is capable of supporting these putative OE neuronal stem cells is FGF8, which appears to be expressed by neuronal progenitor cells within the OE itself (Calof et al., 1997, 1998a; Shou et al., 2000). Calculations of the number of neuronal stem cells in FGF-treated explant cultures suggest that about 1 in 2,500 of the migratory (neuronal) cells in these cultures, is a stem cell (Calof et al., 1998a).

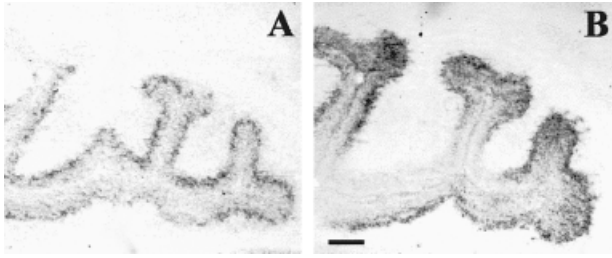


Fig. 3. Expression of *Ngn1* and *Bf-1* in the olfactory epithelium. Non-radioactive in situ hybridization using digoxigenin-labeled cRNA probes was performed on horizontal cryostat sections (20 μm) of fresh-frozen e14.5 mouse OE. Sections were hybridized with antisense RNA probes to (A) *Ngn1* (Ma et al., 1996) and (B) *Bf-1* (Tao and Lai, 1992); sense probe controls (not shown) showed no staining. Hybridization was performed at 60°C in 5 \times SSC, 50% formamide, 50 $\mu\text{g}/\text{ml}$ yeast tRNA, 50 $\mu\text{g}/\text{ml}$ heparin, 0.1% Tween-20, and 5 mM EDTA containing 100 ng/ml of antisense probe. Unbound probe was removed by sequentially washing slides for 10' at 60°C in 1 \times SSC, 1.5 \times SSC, and 2 \times SSC. Slides were treated with RNase (1.0 $\mu\text{g}/\text{ml}$) for 30' at 37°C, washed in 2 \times SSC, and were reacted with sheep anti-DIG AP Fab antibody (1:2,000; Roche). Color was developed using NBT/BCIP as a substrate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, and 0.1% Tween-20 according to the manufacturer's recommendation (Roche). Both *Ngn1* and *Bf-1* are expressed in a similar subset of cells in basal OE. Scale bar = 100 μm .

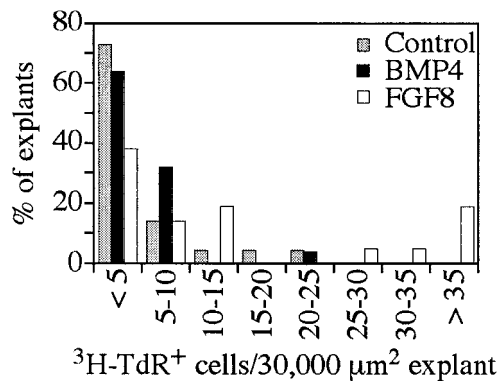


Fig. 4. FGF8 stimulates survival and/or proliferation of putative stem cells in OE explant cultures. Explants of embryonic OE were cultured for a total of 96 hours the presence of BMP4 (0.1 ng/ml), FGF8 (50 nM), or no growth factor (Control), and ³H-thymidine was added for the final 24 hours. The number of ³H-thymidine-positive migratory cells surrounding each explant was counted and divided by the area of the explant (measured using NIH Image 1.61). For comparison, labeling indices were normalized to an average explant area of 30,000 μm^2 and explants were binned according to their normalized labeling indices. In BMP4 and control conditions, the majority of explants have very few proliferating progenitors by 96 hours of culture, since virtually all progenitors have undergone neuronal differentiation by this time in culture. In the presence of FGF8, a small percentage of explants has a large number (>35) of proliferating progenitors. This indicates that these explants contain early progenitors (putative stem cells), which continue to give rise to proliferating neuronal progenitors when grown in the presence of FGF8. Adapted from Shou et al. 2000 with permission of the publisher.

In order to examine the properties of the neuronal stem cell in more detail, we designed experiments to purify neuronal progenitor cells and characterize their development in vitro. To accomplish this, the neuronal cell fraction was prepared from embryonic OE and neuronal progenitors purified from it using anti-NCAM antibodies to remove NCAM-expressing ORNs (Mumm et al., 1996). When these purified progenitors are

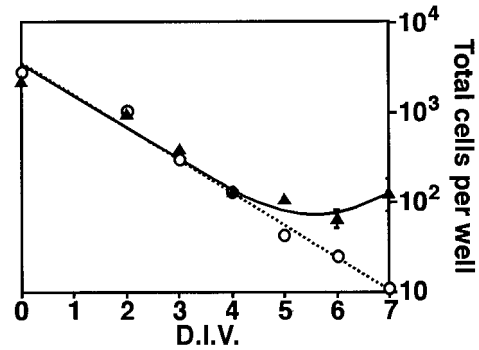


Fig. 5. Emergence of colonies in neuronal colony-forming assays. Purified OE neuronal progenitors were plated onto poly-D-lysine/merosin substrata (dashed lines, o) or monolayers of OE stroma (solid lines, ▲) in 96-well tissue culture plates. Cultures were fixed and processed to detect colonies at 4 hours, 2 days, and every day thereafter for 7 days in culture. The total number of living progenitor cells was counted in each well. Data points show mean \pm SEM for quadruplicate wells at each time point. The data illustrate how progenitor cell numbers decline for the first 4 days in neuronal colony-forming assays, as most purified progenitors undergo differentiation into ORNs, which then rapidly die. If progenitors are cultured on stromal cell feeder layers, however, a small subset survives and generates colonies of cells, which begin to emerge at 5 days in vitro. Approximately 1 of 3,600 initially-plated progenitors gives rise to neuronal colony, suggesting that the frequency of neuronal colony-forming cells (putative stem cells) is about 0.03% of the progenitor cell population. Adapted from Mumm et al. (1996) with permission of the publisher.

grown on feeder layers of cells derived from the stroma that underlies the OE, a small fraction of them survives, proliferates, and generates more undifferentiated progenitors, as well as differentiated ORNs, for at least 2 weeks in culture (Fig. 5; Calof et al., 1998b; Mumm et al., 1996). About 1 in 3,600 originally plated progenitors gives rise to these neuronal colonies, which are illustrated in Figure 6. Based on their ability to undergo continual neurogenesis, as well as their calculated frequency (close to that calculated for FGF-treated OE explants, above, as well as for stem cells isolated from bone marrow; Spooner et al., 1985), we have hypothesized that these neuronal colony-forming cells are the neuronal stem cells of the OE.

An interesting outcome of these studies was the finding that stromal cells provide factors required for neuronal colony development (Mumm et al., 1996). In vitro growth and differentiation of stem cells in several different tissues is known to be dependent on factor(s) made by their associated stromas (e.g., Jones and Watt, 1993). Some progress toward identification of the pro-neurogenic factor(s) produced by OE stroma is beginning to be made. For example, media conditioned over cultured stromal cells has been shown to stimulate neuronal colony formation, and bone morphogenetic proteins (BMPs) may play a crucial role in the ability of OE stroma to support neurogenesis (Crocker et al., 2000; Shou et al., 2000). It is intriguing to speculate that the continued proximity of neuronal progenitor cells of the OE to their stroma might be an important factor underlying the ability of the OE to maintain its neuronal stem cells and regenerate neurons. The identification of the molecules in stromal cell conditioned medium that promote neurogenesis should provide invaluable information about this important question.

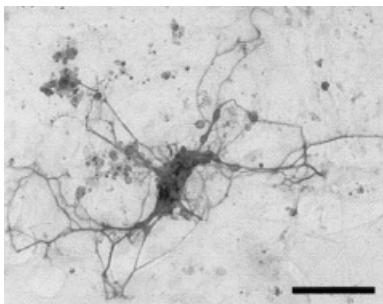


Fig. 6. Typical neuronal colony from a colony-forming assay. Purified OE progenitors were cultured over monolayers of growth-arrested stroma cells in 96-well plates according to published procedures (Mumm et al., 1996; Shou et al., 1999b). After 6 days in vitro, cultures were fixed (15 min with 3.7% formaldehyde/5% sucrose in phosphate-buffered saline) and processed for NCAM immunoreactivity to reveal neuronal colonies. Cultures were incubated with undiluted H28 rat anti-NCAM hybridoma supernatant (Gennarini et al., 1984) and primary antibody detected with horseradish peroxidase-conjugated goat anti-rat IgG (Jackson, 1:500 dilution). The neuronal colony shown here contains many (>30) ORNs, bearing extensive neurites tipped with growth cones. Scale bar = 50 μ m (Bonnin and Calof, unpublished results).

MASH1-Expressing Neuronal Progenitor

The first evidence that the bHLH transcription factor, MASH1 (Mammalian Achaete Scute Homologue 1) is expressed at an important stage in OE neurogenesis came from experiments on the *Mash1* gene in mice. *Mash1* is expressed in subsets of neural progenitors in the central and peripheral nervous systems, including the olfactory epithelium (Guillemot and Joyner, 1993; Johnson et al., 1990; Lo et al., 1991). In mice homozygous for targeted disruption of the *Mash1* gene, profound reductions in the numbers of several types of neurons, including autonomic, enteric, and olfactory receptor neurons, are observed (Guillemot et al., 1993). In the OE of *Mash1*^{-/-} mice, ORN progenitors fail to differentiate into neurons, except in a small ventrocaudal region that is unaffected by loss of *Mash1* function (Cau et al., 1997; Guillemot et al., 1993). Although a few cells that express *Mash1* transcripts are found in the OE of nullizygotes, the OE is abnormally thin and exhibits an abnormally high rate of apoptosis in these animals (Calof et al., 1996b, 1998a; Cau et al., 1997). All the apoptotic cells in *Mash1*^{-/-} OE are NCAM-negative (indicating that apoptosis is not occurring in differentiated ORNs), and no increase in the size of any non-neuronal cell population is apparent. In total, such findings have been interpreted to suggest that neuronal stem cells are still present in *Mash1*^{-/-} OE and produce neuronal progenitor cells (which normally would be MASH1+), but most progenitor cells die without generating ORNs (Calof et al., 1996b, 1998a; Cau et al., 1997; Gordon et al., 1995; Guillemot et al., 1993).

Consistent with the idea that *Mash1* gene activity is required in a precursor of ORNs, and with the observation that genesis of ORNs continues throughout life, MASH1-expressing progenitors also persist throughout life in the OE. MASH1+ cells are detectable in adult OE, although they occur much less frequently than in the OE of embryos; expression of MASH1 in the OE of an adult mouse is shown in Figure 7A. Detailed studies by Gordon et al. (1995) using ³H-thymidine

incorporation combined with MASH1 immunocytochemistry revealed that MASH1+ cells undergo a wave of proliferation following unilateral olfactory bulbectomy (OBX), a procedure that induces apoptosis of ORNs and subsequent regeneration of the OE (Gordon et al., 1995; Holcomb et al., 1995). As shown Figure 7, there is a rapid increase in the number of MASH1-expressing cells in the OE on the operated side, which peaks 6 days post-OBX at a level eight-fold over that in unoperated OE. The number of MASH1-expressing progenitors then declines, and a new steady state (still elevated over control levels) is reached about 10 days following surgery (Fig. 7G). The proliferative dynamics of MASH1-expressing cells (surging when neurogenesis is abruptly stimulated, and declining when neurogenesis declines) are characteristic of transit amplifying cells, which expand their numbers in response to mitogenic stimulation by undergoing rounds of symmetric divisions. In contrast, stem cells, as a consequence of their self-renewal, tend not to undergo such substantial changes in number even when stimulated to proliferate (Hall and Watt, 1989; Potten and Loeffler, 1990). Taken together with data indicating that MASH1+ cells are progenitors of INPs in vivo (see Fig. 9; Gordon et al., 1995), these observations indicate that MASH1-expressing neuronal progenitors are interposed between stem cells and INPs in the ORN lineage, and act like neuronal transit amplifying cells of the OE.

To characterize the MASH1-expressing cell in vitro, OE explant cultures have been used. As shown Figure 8, in OE explants cultured for 8 hours, MASH1-immunoreactive cells constitute about 8% of total migratory cells surrounding each explant (Shou et al., 1999b). MASH1+ cells differ from INPs in their morphology (they are preferentially found in distinctive cell clusters in vitro) and in their numbers (which are 10–15% of that of INPs, both in vivo and in vitro), and their proliferation dynamics in vivo indicates that they divide to give rise to MASH1-negative INPs (Gordon et al., 1995). Thus, under conditions in which rates of OE neurogenesis are manipulated—in vitro and in vivo—the numbers and proliferative states of MASH1+ cells and INPs suggest a precursor-progeny relationship.

Because MASH1-expressing cells are responsive to changes in OE neurogenesis, and because the absence of *Mash1* function disrupts this process, MASH1+ progenitors appear to constitute an important control point for neurogenesis in the ORN lineage. Indeed, recent studies have shown that bone morphogenetic proteins (BMPs) inhibit OE neurogenesis via specific actions exerted on MASH1-expressing neuronal progenitors. Studies by Shou et al. have shown that high concentrations (10–20 ng/ml) of BMPs 2, 4, or 7 block progenitor cell proliferation and generation of ORNs by targeting MASH1 for proteasome-mediated proteolysis (Shou et al., 1999b). As shown in Figure 8, when OE explant cultures are treated with these BMPs, MASH1 immunoreactivity is abolished in neuronal progenitor cells. Moreover, neuronal colony development is completely inhibited in cultures treated with high concentrations of BMP (Shou et al., 1999b). As in *Mash1*^{-/-} animals, there is both a marked decrease in the overall number of progenitor cells, and a substantial increase in the number of apoptotic cells, in BMP-treated OE in vitro. Together, these observations suggest that de-

struction of MASH1 accounts for the anti-neurogenic effect of these BMPs.

Our recent studies have concentrated on identifying the signaling mechanisms that underlie the MASH1-mediated anti-neurogenic effects of BMPs. Genetic studies of *Caenorhabditis elegans* and *Drosophila* support the idea that the identity of the Type I BMP receptor that is activated may dictate the nature of a cell's response to BMPs (Krishna et al., 1999; Nguyen et al., 1998). It is known that the Type IA and IB serine-threonine kinase BMP receptors are expressed in the OE, and the OE is one of the few regions in the developing nervous system to express high levels of the Type IB BMP receptor (Dewulf et al., 1995; Zhang et

al., 1998). We have used OE cultures generated from transgenic mice in which the *BmprIB* gene has been "knocked out" to determine if this receptor is required for the anti-neurogenic effect of BMP2, 4, and 7 (Bonnin et al., 2000; Yi et al., 2000). Our results indicate that treatment with BMP7 fails to cause MASH1 degradation in ORN progenitors cultured from *BmprIB*^{-/-} embryos, whereas BMP4 still induces MASH1 degradation in these cells. Moreover, BMP7's ability to block neurogenesis is also eliminated in cultures of *BmprIB*^{-/-} progenitors tested in neuronal colony-forming assays (Bonnin et al., 2000; Bonnin and Calof, unpublished observations). These results indicate that the anti-neurogenic effect of BMP7 on OE requires BMPRIIB, but that signaling through BMPRIA alone can mediate the anti-neurogenic effect of BMP4. Thus, these findings suggest that signaling by distinct BMPs, acting through different Type I receptors, converges at the level of MASH1-expressing progenitors to regulate neurogenesis in the OE.

Immediate Neuronal Precursor

Studies *in vivo* have shown that the immediate progenitors of ORNs, also referred to as Immediate Neuronal Precursors (INPs), are located in the basal compartment of the OE and have placed INPs among the cells called globose basal cells of the OE (Caggiano et al., 1994; Calof and Chikaraishi, 1989; Mackay-Sim and Kittel, 1991). As described above, MASH1-expressing progenitors are also located in the basal compartment of the OE, and studies *in vivo* were required to understand the relationship between these two cell types. In one study, neurogenesis was induced in adult OE by unilateral olfactory bulbectomy (OBX), and the numbers and proliferative states of MASH1-expressing progenitors and INPs during neurogenesis were followed for several weeks post-surgery (Gordon et al., 1995). The results of these experiments are illustrated in Figure 9. The peak of proliferation by the MASH1+ cell population occurs 24 hours prior to that of INPs, indicating that MASH1-expressing progenitors precede INPs in the ORN developmental pathway.

Genetic studies also indicate that MASH1+ progenitors lie upstream of INPs, and have further identified

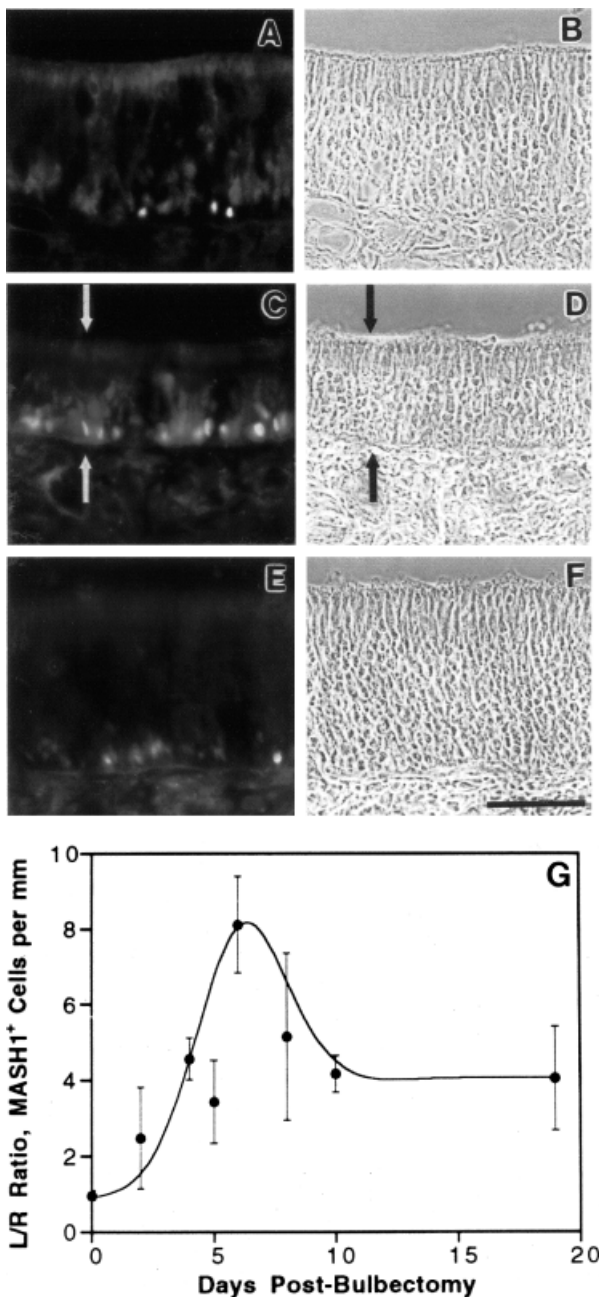


Fig. 7. MASH1 expression in adult olfactory epithelium. **A, B:** OE of an unoperated animal shows rare MASH1-expressing cells in the basal compartment. **C, D:** OE from operated side of a mouse subjected to unilateral bulbectomy (OBX) and sacrificed 5 days post-surgery, processed for MASH1 immunoreactivity. **E, F:** OE from unoperated (control) side of same animal shown in **C** and **D**. The photos show a marked increase in the number of MASH1-positive cells on the ipsilateral side of the operated animal (**C**), compared to contralateral (**E**) or unoperated (**A**) controls. Also apparent is the decrease in overall thickness of the OE, the result of ORN loss, on the ipsilateral side of the operated animal (arrows, **C** and **D**), when compared to either of the controls. Scale bar = 50 μ m. **G:** The number of MASH1-positive cells per mm of OE was determined on both sides for animals sacrificed at various times following unilateral OBX, and data were normalized to account for inter-animal variability. The data are plotted as a function of time following bulbectomy. The data show that, following OBX, there is a rapid increase in the number of MASH1-positive cells in the OE on the operated side. The number of MASH1-expressing cells reaches a peak 6 days after OBX, and then declines by 10 days to a new steady state, which is still elevated over that observed in the control side of the animal. Thus, MASH1-expressing cells have a dynamic response when neurogenesis is induced in the olfactory epithelium. Adapted from (Gordon et al. 1995) with permission of the publisher.

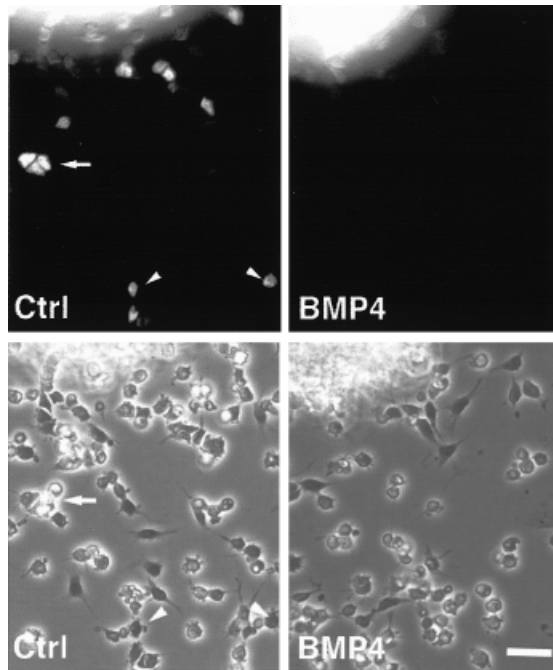


Fig. 8. BMP4 acts to decrease neurogenesis by causing loss of MASH1 protein. OE explants were cultured for 6 hours in vitro, then exposed to BMP4 (20 ng per ml) or to vehicle (Ctrl; culture medium) for an additional 2 hours (total, 8 hours in culture). Cultures were fixed and stained with a mouse anti-MASH1 antibody (Lo et al., 1991). Primary antibody was visualized with rabbit anti-mouse IgG1 followed by Texas red-conjugated goat anti-rabbit IgG (see Gordon et al., 1995). In control conditions (Ctrl), arrow/arrowheads indicate many migratory neuronal progenitor cells expressing MASH1. In BMP4, no cells have detectable MASH1 immunofluorescence. Thus, progenitors exposed to BMP4 rapidly (< 2 hours) lose MASH1, a transcription factor known to be required for ORN production. This disappearance is due to BMP-induced degradation, via the proteasome pathway, of MASH1 protein, which results in premature termination of OE neuronal lineage. Scale bar = 20 μm . Adapted from Shou et al. (1999a) with permission of the publisher.

a potential molecular marker for INPs: the bHLH transcription factor, Neurogenin 1 (*Ngn1*). In situ hybridization shows that *Ngn1* mRNA is expressed in the basal compartment of the OE in midgestation (e 14.5) mouse embryos, indicative of its expression by neuronal progenitors (Sommer et al., 1996; Fig. 3). Interestingly, in *Mash1*^{-/-} mice, neurogenesis is halted and *Ngn1* fails to be expressed (Cau et al., 1997). These findings, taken together with other studies showing that neuronal progenitors undergo apoptosis in *Mash1*^{-/-} OE (Calof et al., 1996a), indicate that INPs are not produced when MASH1 is absent and suggest that *Ngn1* is a marker for INPs. In addition to *Ngn1*, several other molecular markers for INPs have been proposed. These are listed in Table 1.

Initially, using explant cultures of OE purified from e14–15 mouse embryos, the INP was characterized as a proliferating cell that divides symmetrically to give rise to two daughter cells; these daughter cells then rapidly undergo terminal differentiation and begin expressing NCAM, the neuronal cell adhesion molecule expressed exclusively on ORNs within the OE (Calof and Chikaraishi, 1989). Further studies in vitro have confirmed that INPs are the progeny of MASH1-ex-

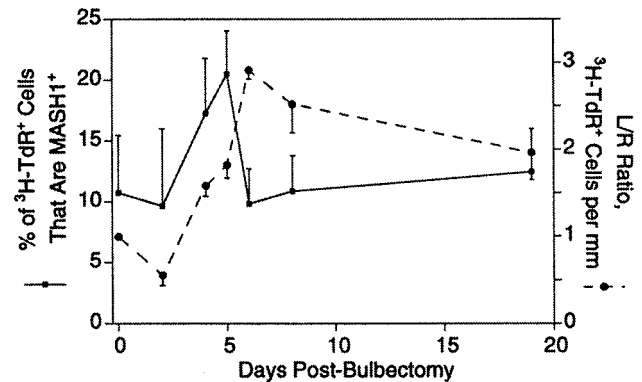


Fig. 9. MASH1 + cells proliferate prior to INPs when neurogenesis is induced in vivo. Adult male mice were subjected to unilateral bulbectomy (OBX) and sacrificed at various times from 2 to 19 days post-surgery. Unoperated control animals were also examined. Animals were given two sequential injections of ³H-Thymidine at 2 and 1 hours prior to sacrifice, in order to label cells in S-phase with high sensitivity. Cryostat sections (12 μm) were taken and processed for autoradiography and MASH1 immunoreactivity. The number of cells per millimeter of basal OE that were MASH1+, had incorporated ³H-Thymidine, or were both MASH1+ and ³H-Thymidine+, was counted. The dashed line shows the number of ³H-Thymidine+ cells/mm in the OE on the operated (OBX) side, normalized to the number of ³H-Thymidine+ cells/mm on the unoperated (contralateral) side. This provides a measure of overall proliferation in the OE on the bulbectomized side. The solid line shows, for the bulbectomized side of each animal, the percentage of ³H-Thymidine+ cells that is also MASH1+; this provides a measure of the relative contribution of MASH1+ cells to overall proliferation. In both cases, the data points represent mean \pm SEM. The data show that peak proliferation of MASH1 + cells precedes that of INPs (which account for the bulk of proliferating cells in basal OE) by approximately 24 hours. Adapted from Gordon et al. (1995) with permission of the publisher.

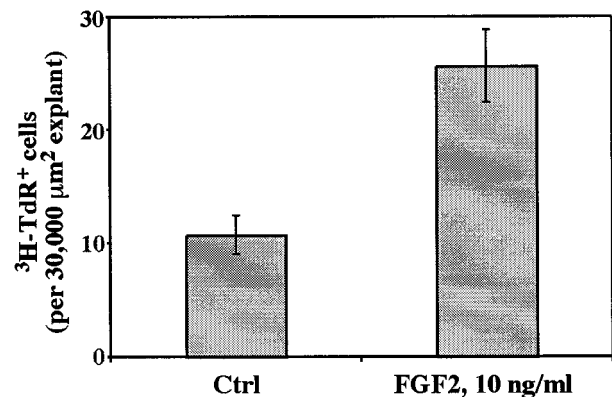


Fig. 10. FGF stimulation of INP divisions in vitro. OE explants were cultured for a total of 48 hours in the presence or absence of 10 ng/ml FGF2, with 0.1 $\mu\text{Ci/ml}$ ³H-thymidine added for the final 24 hours to detect proliferation of INPs. To evaluate labeling indices of INPs, the number of ³H-thymidine+ migratory cells surrounding each explant was counted and divided by the area of the explant. For comparison, labeling indices were normalized to an average explant area of 30,000 μm^2 . Data are expressed as mean \pm SEM for 30 explants in each condition. The data show that the number of proliferating INPs present at 48 hours in vitro is increased 2–3-fold in OE explants cultured in the presence of FGF2. Adapted from Shou et al. (2000) with permission of the publisher.

pressing neuronal progenitors (Gordon et al., 1995). Thus, in explant cultures, INPs can be identified as MASH1-negative, NCAM-negative round cells that incorporate ³H-thymidine and give rise quantitatively to ORNs (Calof and Chikaraishi, 1989; DeHamer et al.,

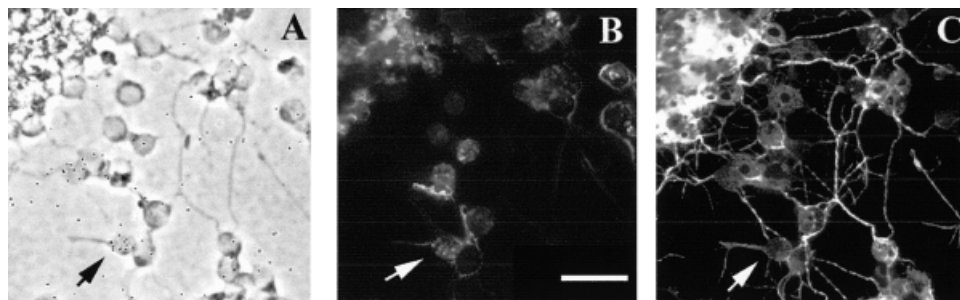


Fig. 11. INPs undergo two successive rounds of cell division *in vitro* to generate ORNs. OE explant cultures were labeled sequentially with two S-phase markers, BrdU and ^3H -thymidine, administered 15 hours apart, so that any double-labeled ORN must be the progeny of a cell that passed through 2 successive S-phases (DeHamer et al., 1994): Briefly, cultures were grown in serum-free medium with BrdU (1:10,000; Amersham cell proliferation reagent no. RPN201) added for the first 6 hours, followed by 15 hours in unlabeled thymidine (50 μM). A second 6-hour pulse of ^3H -thymidine (1 $\mu\text{Ci/ml}$) was then administered, followed by 15 hours more in 50 μM unlabeled thymidine (total time in culture, 42 hours). Cultured cells were fixed with acetone (5 min at room temperature) and processed for autoradiography and BrdU and NCAM immunocytochemistry. Anti-NCAM

antibody (AGID5; DeHamer et al., 1994) was visualized using Cy2-conjugated goat anti-mouse IgG (Jackson; 1:100 dilution). Rat anti-BrdU (Harlan Sera-Lab clone BU1/75 [ICR1]; 1:500 dilution) was visualized with Texas Red goat anti-rat IgG (Jackson; 1:50 dilution). Arrow indicates a cell labeled with BrdU, ^3H -thymidine, and NCAM; the presence of all three markers indicates that this ORN is the progeny of a cell that went through two rounds of division before undergoing neuronal differentiation. **A:** Phase-contrast optics showing the migratory cells and part of the OE explant. Note silver grains present over cells that have incorporated ^3H -thymidine. **B:** Rhodamine optics showing BrdU immunoreactivity. **C:** FITC optics showing NCAM immunoreactivity. Scale bar = 10 μm (Crocker and Calof, unpublished results).

1994). Together, these studies define the INP as a committed neuronal progenitor cell capable of undergoing a limited number of divisions before its progeny undergo terminal differentiation into ORNs. Like the MASH1-expressing neuronal progenitor, then, the INP functions as a neuronal transit amplifying cell in the ORN lineage.

The ability of transit amplifying cells to be stimulated to undergo increased divisions in response to exogenous factors has been described for several lineages (Hall and Watt, 1989; Potten and Loeffler, 1990). By screening for molecules that prolong INP proliferation in OE cultures, several members of the FGF family have been identified as stimulators of INP divisions (Calof et al., 1997; DeHamer et al., 1994; Fig. 10). That multiple divisions of INPs occur in FGFs was demonstrated conclusively in experiments in which ORNs that were generated as a result of two successive rounds of cell division were identified by sequential labeling with bromodeoxyuridine (BrdU) and ^3H -thymidine; such an experiment is illustrated in Figure 11. It was found that the incidence of such double-labeled ORNs is four to five times greater in FGF-treated cultures than in untreated controls while the ultimate neuronal fate of INPs remains unchanged even with FGFs present (DeHamer et al., 1994).

Our current best candidate as the endogenous FGF that regulates INP divisions in the OE is FGF8 (Calof et al., 1997). *In situ* hybridization studies suggest that *fgf8* is expressed by neuronal progenitors, *in vitro* in OE cultures, and *in vivo* in both embryonic and adult OE (Calof et al., 1997). Importantly, FGF8, like FGF2, is able to promote neurogenesis of INPs in OE cultures (Calof et al., 1997). Altogether, these observations suggest that FGF8 may be an endogenous stimulatory factor whose signaling is important for continual neurogenesis in the OE. These findings also suggest that FGF8 expression may be the basis of an autocrine loop in the OE, in which neuronal progenitors respond to a growth factor that they themselves produce.

Recent evidence from our laboratory indicates that several BMPs may regulate proliferation and differentiation

of INPs as well. For example, BMP12 (GDF7), which is expressed in OE stromal cells, stimulates proliferation of INPs in OE cultures (Crocker et al., 2000). Another novel BMP, BMP11, is highly expressed in OE proper (Nakashima et al., 1999; Wu et al., 2001; Wu and Calof, unpublished data). BMP11 belongs to a class of BMPs that includes the negative regulator of muscle growth, Myostatin (GDF8; McPherron et al., 1999). We have recently begun testing for potential negative effects of BMP11 on OE neurogenesis, using GDF8 as a pharmacological substitute (the mature proteins are 90% identical; McPherron et al., 1999). GDF8 strongly inhibits neurogenesis in OE cultures. Unlike BMPs 2, 4, and 7, however, GDF8 treatment has no effect on MASH1+ progenitors; instead, experiments with OE cultured from *Ngn1*-GFP reporter mice suggest that GDF8 inhibits development of *Ngn1*-expressing INPs (Wu et al., 2001; Wu and Calof, unpublished results). These observations suggest that BMP11 may be an endogenous negative regulator of OE neurogenesis, and point out the importance of negative as well as positive regulation of olfactory neurogenesis at the INP stage.

UNDERSTANDING MOLECULAR REGULATION OF NEUROGENESIS IN THE OLFACTORY EPITHELIUM Feedback Regulation of Neurogenesis

Experimental upregulation of neurogenesis in OE by olfactory bulbectomy (OBX) reveals a temporal relationship between ORN death, cell degeneration, progenitor cell proliferation, and generation of new ORNs: This is illustrated in Figure 12. Unilateral OBX causes ORNs in the OE on the operated side to rapidly undergo apoptosis (Holcomb et al., 1995). Neuronal progenitor cells in the ipsilateral OE respond by increasing proliferation, which reaches a peak at about 5–6 days post-bulbectomy (e.g., Gordon et al., 1995; Schwartz Levey et al., 1991). There is marked degeneration of the ORN cell layer, which can be quantified by measuring epithelial thickness, and this degeneration follows a timecourse similar to that of progenitor cell proliferation: epithelial thickness is at a minimum

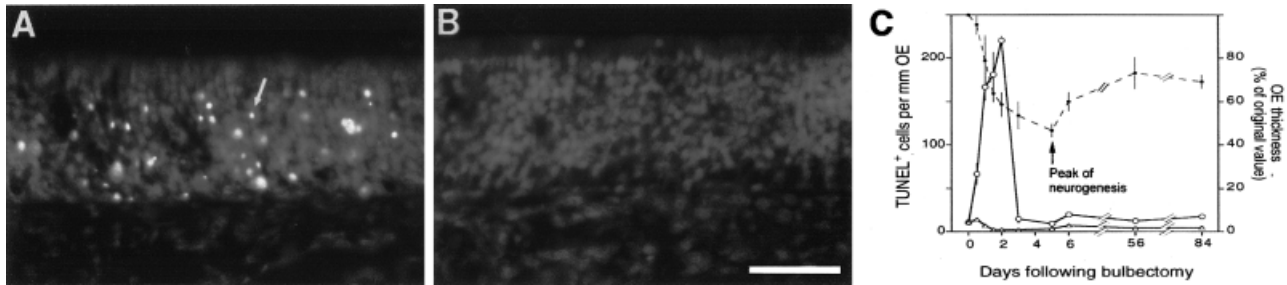


Fig. 12. Relationship between neuronal death and neurogenesis in the olfactory epithelium. In vivo, unilateral OBX rapidly induces apoptosis of neuronal cells in the OE, which can be visualized using the TUNEL technique (Gavrieli et al., 1992); (Holcomb et al., 1995). **A:** OE from the bullectomized side of a mouse sacrificed 1 day post-surgery. **B:** OE from the contralateral (control) side of the same animal. The arrow in A points to a brightly fluorescent nucleus of a cell undergoing apoptosis. Scale bar = 50 μ m. **C:** Time course of DNA fragmentation and changes in epithelial thickness following unilateral bullectomy. Numbers of TUNEL+ cells/mm septal OE are plotted

(open circles, solid line = OBX side; open triangles, solid line = contralateral unoperated side), together with changes in the average thickness of the bullectomized OE (solid circles, dashed line). The data show that the peak of apoptosis occurs 2 days following OBX, approximately 3 days prior to the time of maximal OE degeneration (5 days post-OBX). Interestingly, 5 days post-OBX is also the time when OBX-induced proliferation of neuronal progenitors peaks in the OE. Adapted from Holcomb et al. (1995) with permission of the publisher.

at about 5 days post-bullectomy (Costanzo and Grazia-dei, 1983; Holcomb et al., 1995; Schwartz Levey et al., 1991). Then, as new ORNs are generated, epithelial thickness increases and progenitor cell proliferation decreases, albeit to a level that is somewhat elevated over that seen in unoperated OE (Gordon et al., 1995; Holcomb et al., 1995; Schwartz Levey et al., 1991). The striking observation that the peak of neurogenesis corresponds with a maximum loss of ORNs suggests that somehow, neuronal progenitors “read” the number of differentiated neurons in their immediate environment and regulate the production of new neurons accordingly. Thus, neurogenesis is downregulated when ORN numbers are high, and upregulated when ORN numbers are low.

Observations in vitro suggest that this process, which we have called feedback inhibition of neurogenesis, is mediated by molecular signals produced by ORNs. This was first revealed in studies using neuronal colony-forming assays. We found that when purified OE neuronal progenitors were grown in the presence of a 20-fold excess of differentiated ORNs, the formation of neuronal colonies was inhibited three- to four-fold (Mumm et al., 1996). Moreover, experiments in which ORNs were first heat-treated prior to adding them to colony-forming assays indicated that an ORN-derived, heat-labile macromolecule was responsible for this effect (Calof et al., 1998b). Thus, the results of tissue culture experiments indicated that differentiated ORNs produce molecular signal(s) that feed back to inhibit production of new neurons by their own progenitors. Such a phenomenon could explain the surge in neurogenesis in the OE in vivo that occurs as a consequence of ORN death following bullectomy: the post-OBX increase in neurogenesis could be due to loss of an inhibitory signal normally produced by living ORNs.

What molecules might be responsible for ORN-mediated feedback inhibition of OE neurogenesis? Our studies suggest that BMP4 may play a role in this process. In addition to having an inhibitory effect on OE neurogenesis at high concentrations (10–20 ng/ml) in vitro (see Fig. 8), we have recently found that, at much lower concentrations (0.1–0.2 ng/ml), BMP4 selectively promotes the survival of newly-differentiated ORNs (Shou

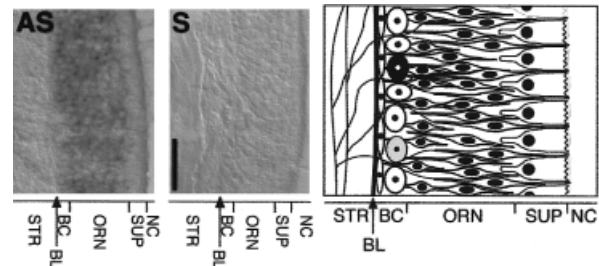


Fig. 13. Localization of *Bmp4* transcripts in adult mouse olfactory epithelium. In situ hybridization to cryostat sections of adult mouse OE using antisense (AS) or sense (control; S) probes shows that *Bmp4* transcripts are located in the ORN layer. Scale bar = 50 μ m. **Right:** The major cell layers of the OE. OE proper is separated from its underlying stroma (STR) by a basal lamina (BL). Within the basal cell layers (BC) of the OE, a layer of flat horizontal basal cells underlines the globose neuronal progenitors, which include stem cells (black), MASH1 + progenitors (gray), and INPs (white). Several layers of postmitotic olfactory receptor neurons (ORN) lie atop the globose progenitor cells and are capped by the supporting cell layer (SUP), which is adjacent to the nasal cavity (NC). Adapted from Shou et al. (2000) with permission of the publisher.

et al., 2000). Importantly, in adult OE, *Bmp4* mRNA is expressed within the neuronal layers, strongly suggesting that ORNs produce BMP4; this is shown in Fig. 13 (Shou et al., 2000). One possibility, then, is that when ORNs are induced to die, the overall level of BMP4 to which progenitors are exposed decreases, since the number of BMP4-producing cells in the OE would thereby be reduced. This decrease in BMP4 might be the signal that permits progenitor cells to increase their proliferation and re-populate the OE with ORNs. Moreover, these newly generated ORNs should begin to produce BMP4, which would in turn serve both to support their own survival, and, as ORN numbers increase, to suppress further progenitor cell proliferation. To test this idea, we have recently begun generating transgenic mouse lines in which a soluble BMP4 antagonist, noggin, is expressed by differentiated ORNs under the control tissue-specific genomic regulatory elements derived from the OMP gene (Danciger

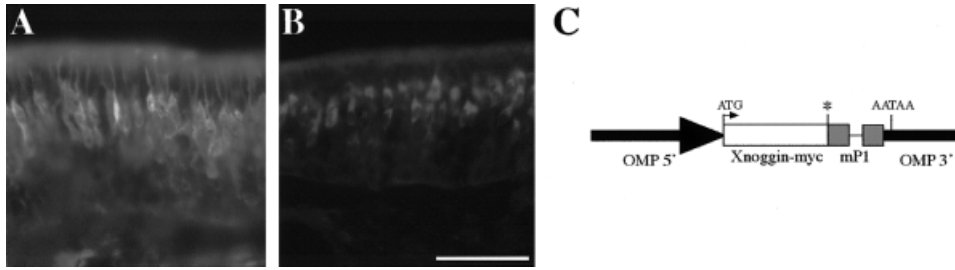


Fig. 14. OMP-Noggin transgenic mice. OMP and transgene expression in the olfactory epithelium of neonatal mice of the OMP-NOG^{57.3} transgenic mouse line. Transgenic mice were generated by pronuclear injection using standard methods (Hogan et al., 1994). Lines were established from 8 founder animals, of which 5 showed transgene expression by RT-PCR. **A:** Olfactory marker protein (OMP) expression. Mice were euthanized and heads fixed by Immersion fixed 4% paraformaldehyde in phosphate buffer for 2 hours at room temperature, and 14- μ m cryostat sections were prepared. Sections were processed for immunohistochemistry using a goat antiserum to OMP (generous gift of Frank Margolis), visualized using FITC-conjugated rabbit anti-goat IgG (1:50; Cappel-ICN). OMP stains mature ORNs within the OE. **B:** Noggin transgene expression. An adjacent section to the one in A was processed for immunohistochemistry using a mouse monoclonal anti-myc antibody (9E10; see Evan et al., 1985) to

detect myc-epitope-tagged noggin transgene. Primary antibody was visualized using a Texas Red-conjugated goat anti-mouse IgG1 (1:50, Southern Biotechnology). Anti-myc immunoreactivity is present in the same ORN layer as OMP immunoreactivity, demonstrating tissue-specific expression driven by the OMP promoter. Scale bar = 50 μ m. **C:** Diagram of the transgenic construct. A 0.9-kb fragment encoding myc-epitope-tagged *Xenopus* noggin (Xnoggin-myc) is expressed under the control of 5 kb of 5' and 3kb of 3' sequence from the rat *Omp* gene (Danciger et al., 1989). Base pairs 95 to 464 of the mouse protamine 1 gene (*mP1*), which includes a 93-bp intron, is included in the construct to help stabilize the mRNA (Peschon et al., 1987). The position of the transcriptional start (ATG), stop (*), and polyadenylation signal (AATAA) are also indicated. Not drawn to scale (Murray and Calof, unpublished results).

et al., 1989; Murray et al., 1998). This is illustrated in Figure 14. Interestingly, expression of the OMP-noggin transgene results in a significant decrease in BrDU incorporation by progenitor cells in the OE of neonatal mice, suggesting that the dynamics of neurogenesis may be changed in these animals (Murray and Calof, 2001; Murray and Calof, unpublished observations). Thus, the idea that BMPs play an important role in regulating neurogenesis *in vivo* appears to be a promising one.

What Are the Early Determinants of Progenitor Cell Identity and Function in the Olfactory Epithelium?

A critical issue that remains for understanding the regulation of olfactory neurogenesis is the identification of the molecular determinants that are important in specifying progenitor cell identity and function when the OE is initially established. One such determinant may be retinoic acid (RA). *In vitro*, RA is known to promote neuronal differentiation and inhibit mesodermal characteristics of several cell types, including ORNs (Bain et al., 1996; LaMantia et al., 2000; Strubing et al., 1995). *In vivo* analyses also suggest that RA signaling is important in olfactory development, since hypoplasia and alterations in apoptosis occur in the frontonasal region when RA is depleted during embryonic development (Dickman et al., 1997). In addition, treatment of e8.5 mouse embryos with citral, a RA synthesis inhibitor, prevents normal invagination and subsequent development of the OE (Anchan et al., 1997). *Pax-6* mutant mice show craniofacial malformations similar to those observed when RA signaling has been depleted during development, including loss of OE and olfactory bulb tissue (Fujiwara et al., 1994; Hill et al., 1992; Hogan et al., 1986; 1988). *Pax-6*^{-/-} mice have remnants of an epithelial structure resembling an "atrophic" OE, but a mature olfactory nerve cannot be identified (Jimenez et al., 2000). Transgenic mice, in which retinoic acid response elements (RAREs) drive

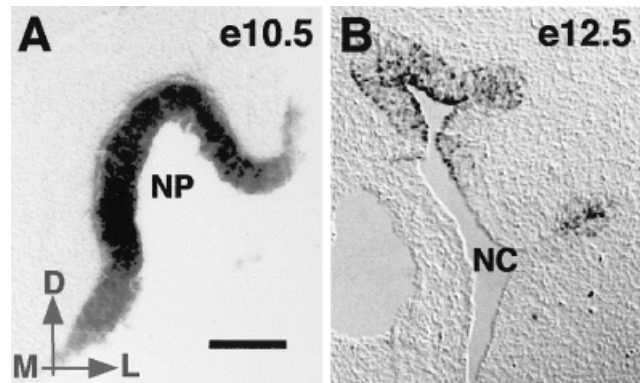


Fig. 15. Expression of *Aldh6* mRNA in developing olfactory epithelia. *In situ* hybridization for *Aldh6* mRNA in coronal sections at 10.5 (A) and 12.5 (B) days of gestation. Embryos were fixed overnight in 4% paraformaldehyde in phosphate buffer, and cryostat sections (20 μ m) were cut. cRNA probes (bp 433–1458 of *Aldh6* cDNA) were labeled with digoxigenin-UTP and hybridization (probe concentration = 20 ng/ml) performed at 65°C in 50% formamide, 5 \times SSC, 0.3 mg/ml yeast tRNA, 100 μ g/ml heparin, 1 \times Denhardt's, 0.1% Tween-20, 0.1% CHAPS, and 5 mM EDTA. Unbound probe was removed with sequential washes in 0.2 \times SSC (3 times at 65°C), followed by room temperature washes in phosphate-buffered saline containing 2 mg/ml BSA and 0.1% Triton-X-100. Alkaline phosphatase-conjugated anti-digoxigenin antibody (sheep Fab, 1:2,000 dilution; Roche) was visualized with BCIP/NBT according to the manufacturer's instructions. Axis: Up is dorsal (D), left is medial (M), and right is lateral (L). NP = nasal pit; NC = nasal cavity. Scale bar = 100 μ m (S Kawauchi and Calof, unpublished results).

expression of a *lacZ* reporter gene, show a positive *lacZ* signal in wildtype developing OE, indicating that RA signaling is normally active in this region. Interestingly, however, this signal is abolished in the absence of *Pax-6* gene function, even though tissues from these animals retain responsiveness to exogenous RA. Taken together, these results suggest that the loss of OE structure observed in *Pax-6* mutants is associated with

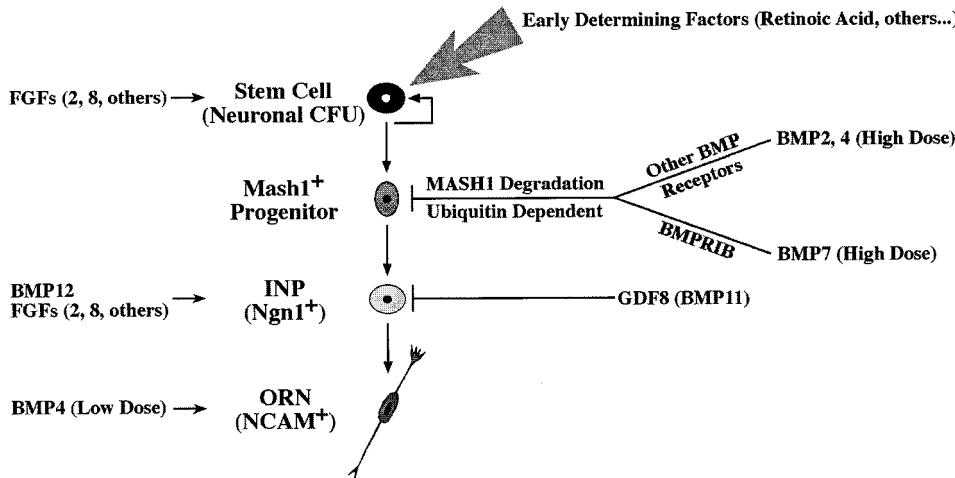


Fig. 16. Molecular regulation of neurogenesis in the ORN lineage. The figure summarizes our current understanding of positive and negative regulation of neurogenesis by extrinsic growth factors. For details, see text.

a loss of retinoid signaling due to absence of local RA synthesis, rather than absence of functional RA receptors (Anchan et al., 1997; Enwright and Grainger, 2000).

Recent studies have shown that RA signaling must be supplied by the activities of retinaldehyde dehydrogenases, which are expressed in tissue-specific patterns (Niederreither et al., 1999). One recently-cloned member of this family, *aldehyde dehydrogenase 6* (*Aldh6*, also known as *Raldh3*), has been shown to be expressed in developing OE and other sensory and neural structures (e.g., neural retina, otic vesicle, mid-brain isthmus, and Rathke's pouch; see Grun et al., 2000; Li et al., 2000; Mic et al., 2000). Interestingly, expression of *Aldh6* is abolished in the facial surface ectoderm covering the eye and in the nasal region in *Pax-6* mutant rat embryos examined at e11.5 (Suzuki et al., 2000). These data suggest that RA signaling is mediated by *Aldh6* and that this activity in the developing OE is dependent upon *Pax-6*. It has been shown that RA can induce *Mash1* in some cell types and that expression of some *Bmps* (*Bmp-2*, *-4*, and *-7*) may be regulated by RA (Bain et al., 1996; Dupe et al., 1999; Helvering et al., 2000). Importantly, both MASH1 and BMPs are known to be required for proper neurogenesis in the OE (see above and Guillemot et al., 1993; Shou et al., 1999b 2000). Thus, RA may be the sought-after critical determinant of neuronal progenitor cell identity and function in the developing olfactory epithelium.

To begin to address this question, we have examined the expression of retinaldehyde dehydrogenases in the developing OE. In situ hybridization studies of *Aldh6* expression are shown in Figure 15. *Aldh6* is expressed in mouse head ectoderm at e9.5, and there is a strong signal present in invaginating OE at e10.5 (Fig. 15A). At e12.5, *Aldh6* is expressed in the recesses of the developing olfactory turbinates. (Fig. 15B). By e15.5, most *Aldh6* expression appears to have shifted to stromal cells underlying the OE (Grun et al., 2000; data not shown); the OE of older animals has not yet been examined. Since *Aldh6* appears to be the only retinaldehyde dehydrogenase expressed in developing OE (Kawauchi and Calof, unpublished observations), and since *Pax6* regulation of olfactory development appears

to be dependent on RA signaling (as described above), our current hypothesis is that ALDH6 may be the crucial link between *Pax6* and RA signaling in the regulation of progenitor cell identity in the OE.

Regulation of ORN Progenitors by Extrinsic Factors Occurs at Every Cell Stage

Figure 16 summarizes the current state of our knowledge concerning the regulation of olfactory neurogenesis by extrinsic growth factors. Although the number of growth factors that have been identified as playing important roles in this process continues to increase, several principles are emerging. Multiple members of two different growth factor families—the BMPs and the FGFs—appear to play particularly important roles in regulating neurogenesis in the OE. Significantly, at least one of these factors—BMP4—exerts opposing effects on cells at different stages in the ORN lineage, acting to inhibit proliferation of MASH1-expressing progenitors when present at high concentration, while at the same time acting to stimulate survival of newly generated ORNs when present at low concentration. Importantly, regulation of progenitor cell proliferation and/or survival by extrinsic growth factors occurs at every identifiable cell stage. Moreover, this regulation is not always positive; in fact, negative regulation of neurogenesis is clearly of great importance in the olfactory epithelium, a system in which feedback inhibition of neuronal progenitor cell proliferation plays a crucial role in maintaining proper neuron number.

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