Molecular Signals Regulating Proliferation of Stem and Progenitor Cells in Mouse Olfactory Epithelium

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Abstract
To understand how signaling molecules regulate the generation of neurons from proliferating stem cells and neuronal progenitors in the developing and regenerating nervous system, we have studied neurogenesis in a model neurogenic epithelium, the olfactory epithelium (OE) of the mouse. Our studies have employed a candidate approach to test signaling molecules of potential importance in regulating neurogenesis and have utilized methods that include tissue culture, in situ hybridization and mouse genetics. Using these approaches, we have identified three distinct stages of stem and transit amplifying progenitor cells in the differentiation pathway of olfactory receptor neurons (ORNs) and have identified mechanisms by which the development of each of these progenitor cell types is regulated by signals produced both within the OE itself and by its underlying stroma. Our results indicate that regulation of olfactory neurogenesis is critically dependent on multiple signaling molecules from two different polypeptide growth factor superfamilies, the fibroblast growth factors and the transforming growth factor β (TGF-β) group. In addition, they indicate that these signaling molecules interact in at least two important ways: first, opposing signals converge on cells at specific developmental stages in the ORN pathway to regulate proliferation and differentiation; and second, these signaling molecules – particularly the TGF-βs and their antagonists – play key roles in feedback loops that regulate the size of progenitor cell pools and thereby neuron number, during development and regeneration.

Key Words
Neurogenesis · Olfactory epithelium · Bone morphogenetic protein · Fibroblast growth factor · Stem cell · Neuronal progenitor · Growth/differentiation factor · Follistatin

Introduction
In order to understand basic principles that govern the generation of neurons in the mammalian nervous system, we have studied the molecular regulation of neurogenesis in a model neurogenic epithelium, the olfactory epithelium (OE) of the mouse. The OE is morphologically and functionally similar to the embryonic neuroepithelia that generate the rest of the nervous system, but has significant advantages as a system for study. First, the OE is simpler,
producing only one major type of neuron, the olfactory receptor neuron (ORN). Second, the OE retains both its epithelial morphology and the ability to generate neurons throughout life [1]. Figure 1A shows the spatial relationships between ORNs, the neuronal progenitor cells that underlie them in the OE and the connective tissue stroma that lies beneath the basal lamina of the OE [2].

Tissue culture assays have provided a powerful approach for exploring molecular mechanisms regulating neurogenesis in mouse OE, allowing identification of four different cell stages in the ORN lineage, which are shown in figure 1B [2]. Such studies have revealed the existence of a self-renewing stem cell: this cell gives rise to neuronal progenitors that express the Mash1 gene, which encodes a basic helix-loop-helix (bHLH) transcription factor known to be essential for proper ORN development [3–6]. Mash1-expressing progenitors in turn give rise to immediate neuronal precursors (INPs), which express a different bHLH transcription factor, neurogenin1 (Ngn1) [7–9]. INPs then divide, giving rise to daughter cells that undergo terminal differentiation into ORNs, which express the neuronal cell adhesion molecule Ncam [10, 11].

Because the OE displays robust neurogenesis, in vitro and in vivo, it has been possible to take clues from these different types of studies to target candidate signaling molecules for investigation concerning their roles as potential regulators of neurogenesis in this system. As described below, these studies have led to the identification of multiple members of two polypeptide growth factor superfamilies, the fibroblast growth factors (FGFs) and the transforming growth factor-β (TGF-β) group, as important regulators of OE neurogenesis. These findings in turn have led us to develop new experimental approaches for expanding our understanding of the roles of stem and progenitor cells in regulating developmental and regenerative neurogenesis.

Fig. 1. A Diagram of the arrangement of cells in the mature OE. Sus = Sustentacular cells, adjacent to the nasal cavity; ORN = olfactory receptor neuron layers; GBC = globose basal cell layer, containing stem cells (white) and neuronal progenitors (Mash1+ progenitors = black; Ngn1+ immediate neuronal precursors, INPs = dark gray); HBC = horizontal basal cell layer; Str = stroma; ON = olfactory nerve (axons of ORNs). B Scheme of the neuronal differentiation pathway in the OE.
Fig. 2. Expression of Raldh1, Raldh2 and Raldh3 in the developing olfactory pit. FB = Forebrain; PR = pigmented retinal epithelium; VR = ventral retina; DR = dorsal retina; M = medial; D = dorsal; L = lateral. A Raldh3 is the only Raldh expressed within the OE at this stage; expression is also observed in the retina. B, C Raldh1 and Raldh2 are not expressed in the OE, although Raldh1 is expressed in the retina. D Mash1, a gene expressed by early OE neuronal progenitors, is expressed in the same region as Raldh3.

Results and Discussion

Potential Role of Retinoid Signaling in OE Neurogenesis

All progenitor cells and neurons of the OE ultimately originate in the olfactory placodes, two oval epithelial thickenings located in the anterolateral portions of the developing head, which appear at about day 9 of gestation (E9.5) in the mouse [12]. About 1 day later, the placodes invaginate to form olfactory pits, which continue to deepen and form progressively more recesses as development proceeds. Markers of the different cell types in the ORN lineage are already apparent by the time olfactory pits are visible [7], and the characteristic pattern of organization and distribution of the cells within the OE begins to become apparent in the mouse around E12.5 [Kawauchi and Calof, unpubl. data]. By E14.5, the different cell layers of the mature OE, characterized by the molecular markers expressed by the cells within these layers, are in place for the most part [8, 10].

To understand early signaling events that specify progenitor cell identity and function when the OE is first established, we have looked at the expression of genes involved in several different signaling systems in the developing olfactory pit. Our attention was drawn to retinoic acid (RA) because RA is known to have widespread functions in vertebrate development, including neural development [13–17]. We thought RA signaling likely to be of special significance in early OE development, since animals in which RA signaling has been depleted show a spectrum of craniofacial malformations, including loss of OE and olfactory bulb tissue, that mimic the defects seen in animals with mutations in Pax6, a neural patterning gene expressed in the olfactory placode [18–25]. Interestingly, expression of RA-related genes cannot be detected in the anterior head region of Pax6 mutant embryos [26, 27].

The critical and rate-limiting step in the biosynthesis of RA is the conversion of the retinaldehyde intermediate (retinal) to active RA by specific cytosolic enzymes, the retinaldehyde dehydrogenases (RALDHs). Tissue-specific expression of RALDHs appears to be the crucial factor in determining where and when RA acts during development [28]. To date, 4 closely related members of the Raldh gene family have been identified (Raldh1–Raldh4; Raldh4, identified in 2003, appears to be restricted in expression to liver and kidney [28, 29]). To determine which Raldh genes are expressed in the developing olfactory pit, we performed in situ hybridization on serial sections of mouse embryo heads at E10.5, a time when the olfactory pit is invaginating. As shown in figure 2, of the 3 Raldh genes tested, only Raldh3 is expressed within the neuroepithelium of the developing OE. Expression can also be seen in the retina, as reported previously [30]. In contrast to Raldh3, neither Raldh1 nor Raldh2 is expressed within the OE, although Raldh1 is expressed in neural retina (fig. 2B). Interestingly, the region of Raldh3 expression encompasses that in which Mash1, a gene expressed by early neuronal progenitor cells in the OE neuronal lineage, is also expressed (fig. 2D [3]).

The coincidence of Raldh3 expression with that of Mash1 suggests that Raldh3 is expressed by neuronal pro-
ger cells within the OE. To characterize the cells that express RALDHs, we have performed preliminary tests using a fluorescent substrate for RALDHs [Bodipy aminoacetaldde (BAAA)]. RALDHs oxidize BAAA in the cytosol and convert it to Bodipy amionoacetate (BA), which is negatively charged and remains trapped within the cell for at least 30 min; BAA can be visualized using a fluorescence microscope equipped with a FITC filter [31]. An antibody panning method was used to separate living neuronal progenitors from Ncam-expressing ORNs in dissociated cell fractions from purified embryonic OE [4], and cells were incubated with BAAA and analyzed. Our observations indicate that the majority of BAA+ cells are present in the NCAM-negative neuronal progenitor cell fraction, rather than in the ORN fraction, suggesting that BAA oxidation activity (and, by extension, Raldh3) is expressed primarily by neuronal progenitors in the OE. Moreover, since BAA+ cells account for only a small percentage of the progenitor cell fraction (less than 10%), they may represent a rare cell type within this fraction, possibly neural stem cells [see below and S. Kawauchi, F. Grün and A.L. Calof, manuscript in preparation]. Thus, the possibility exists that Raldh3 may be a marker for OE neural stem cells, at least at specific developmental ages.

Taken together, these observations suggest a model in which Raldh3, expressed by neural progenitor cells and regulated (directly or indirectly) by Pax6, mediates RA activity in the invaginating olfactory pit; they further imply that this activity is required for proper OE development. Recently, Dupe et al. [32] reported the generation of mice with targeted inactivation of the Raldh3 gene. These animals show aberrant development of the nasolacrimal ducts and morphological defects in the nasal cavity structure; effects on neurogenesis in the OE, if any, have not been reported. Thus, RA signaling via RALDH3 is clearly required for some aspects of olfactory development, but the role of endogenous Raldh3 expressed by progenitor cells in olfactory neurogenesis has yet to be understood. By isolating and culturing RALDH-expressing progenitors, using fluorescence-activated cell sorting in combination with the BAAA fluorescent RALDH substrate and neuronal markers, we hope to gain further understanding of the role of RA signaling in OE neurogenesis.

Strategies for Identification and Isolation of the OE Neural Stem Cell

In mice with targeted inactivation of the Mash1 gene, which encodes a bHLH transcription factor homologous to the Drosophila proneural genes achaete and scute, several types of neurons, including ORNs, fail to develop [6]. Recently, we have performed a detailed study of neurogenesis in the OE of Mash1–/– animals [8] and found that despite the fact that ORNs fail to differentiate, proliferating cells are present in large numbers. Moreover, these cells appear to fill the (aberrantly thin) mutant OE. By performing in situ hybridization analysis using a probe specific for the 3′ untranslated region (UTR) of the Mash1 transcript (this portion of the gene is still present in the targeted allele), we were able to determine that most if not all of the proliferating cells in Mash1–/– OE express this mutant Mash1 transcript. These cells do not express normal Mash1 transcripts nor do they express Ngn1 (an INP marker) or Ncam (an ORN marker). Interestingly, however, they do express Steel, a marker for sustentacular cells, the supporting cells that line the apical surface of the OE [6, 8]. These findings are illustrated in figure 3. Thus, in the absence of Mash1 function, the OE becomes populated by proliferating cells that share characteristics of both neuronal progenitors (ability to proliferate and expression of Mash1′ UTR) and sustentacular cells (expression of Steel). Our interpretation of this finding is that many of the proliferating cells in the OE of Mash1–/– embryos can be thought of as being ‘frozen’ at an early stage in the neuronal differentiation pathway. In this model, expression by these cells of both Steel and the Mash1′ UTR is indicative of an early multipotent state, implying that these cells may be neural stem cells. Under normal conditions, the progeny of these cells would become restricted to a neuronal fate (i.e. become ORNs, via MASH1+ cells which then give rise to INPs) or to a glial (sustentacular, Steel+ only) fate. Normal MASH1 protein must be required for proper neuronal determination in these cells, and in the absence of MASH1 (as in Mash1–/– OE), these cells accumulate and ultimately undergo apoptosis [8].

We have now begun to use expansion of gene expression in Mash1–/– OE as an assay for identifying genes expressed by the putative OE neural stem cell. To test this idea, we have analyzed expression of two genes expressed in developing OE, Raldh3 and Sox2. Sox2 is an SRY transcription factor of the Sox family; these factors are likely candidates for stem cell markers, since they are expressed throughout the neural primordium [33], are important regulators of embryonic development [34] and have been shown to direct neural progenitor cell identity [35–37]. In particular, expression of Sox2 is associated with uncommitted stem cells [38] and has been shown to be downregulated as neuronal progenitor cells mature (possibly through the action of proneural genes such as Mash1 [36]).
Fig. 3. Expression of Ncam, Steel and Mash1 3’UTR in the OE of wild-type and Mash1−/− embryos. SUS = Sustentacular cells; BL = basal layer. Nonradioactive in situ hybridization using digoxigenin-labeled cRNA probes was performed on horizontal cryostat sections (20 µm) of paraformaldehyde-fixed E14.5 (A–F) and E17.5 (G–L) mouse OE from wild-type (A–C and G–I) and Mash1−/− (D–F and J–L) littermates. Sections were hybridized with antisense RNA probes to Ncam (A, D, G, J), Steel (B, E, H, K) and Mash1 3’ UTR (C, F, I, L). Ncam is expressed in ORNs, Steel is expressed in sustentacular cells, and 3’ Mash1 is expressed in OE neuronal progenitors (A–C and G–I). In Mash1−/− embryos, Ncam expression is almost completely absent while Steel and 3’ Mash1 appear to be expressed in virtually all cells in the epithelium (D–F and J–L). Scale bar in F (for A–F) = 10 µm; in L (for G–L) = 10 µm. Reproduced with permission (copyright 2003, Society for Neuroscience).
Figure 4. Distribution of Mash1 3’ UTR, Sox2 and Raldh3 mRNA in wild-type and Mash1−/− littermate embryos at E14.5 in the OE. White asterisks (*) indicate the OE proper; Str = stroma underlying the OE in the nasal region. A, C, E = wild type; B, D, F = Mash1−/−. Note that the OE is thinner in Mash1−/− embryos, and all three transcripts are now expressed in cells throughout the OE, rather than being concentrated in basal and apical layers. Scale bar = 100 μm.

Figure 4 shows in situ hybridization analysis of expression of Sox2 and Raldh3, as well as Mash1 3’ UTR, in OE of E14.5 Mash1−/− animals and their wild-type littermates. In normal animals, expression of both Sox2 and Raldh3 mimics generally that of Mash1, with a higher density of expressing cells in the basal and apical layers of the epithelium. However, in Mash1−/− OE, expression of both Sox2 and Raldh3 expands to encompass most of the cells in the OE (white asterisks), as does expression of the Mash1 3’ UTR. These observations suggest that both Raldh3 and Sox2 may be expressed by OE neural stem cells, as well as by the committed neuronal progenitors that express Mash1 in normal OE [3].

How can we use these observations to help us isolate and study OE neural stem cells? Most methods for isolating stem cells, including our own, have relied largely upon negative selection criteria, using the absence of known stage-specific markers as evidence of a cell’s early position in a differentiation pathway [4]. A more direct and accurate approach is to use markers that are expressed specifically by the stem cells themselves. Two factors are directing our approach to test the hypothesis that Sox2 is a marker for OE neural stem cells: (1) the abundance of evidence suggesting that Sox2 is expressed by stem cells in many regions (fig. 4) [35–39] and (2) availability of a transgenic mouse, in which a β-galactosidase/neomycin phosphotransferase fusion gene (beta-geo) is expressed in the Sox2 domain, thus allowing Sox2-expressing cells to be identified on the basis of their expression of the β-galactosidase reporter and selected for in tissue culture by growth in the antibiotic G418 [35, 38–40].
We have first investigated expression of the Sox2-beta-geo transgene in the OE of hemizygous animals at E12.5 (homozygotes die shortly after implantation [38]). The results are shown in figure 5. Using X-gal staining to detect transgene expression, we find a high concentration of heavily stained cells in the apical layer of the OE, as well as in the basal region. Interestingly, it is known that at this early stage the majority of cells undergoing mitosis are located in the apical layer of the epithelium [41], suggesting that these X-gal+ cells are in fact dividing, undifferentiated progenitor cells, as predicted.

To gain further insight into the characteristics of the OE neural stem cell, we are performing experiments to test the hypotheses that (a) Sox2-expressing cells are indeed neural stem cells of the OE and (b) the level of Mash1 expression regulates the progress of OE neural stem cells through a neuronal versus glial (sustentacular) differentiation program. We are using a genetic approach to introduce the Sox2-beta-geo fusion gene onto a Mash1–/– background. Using OE isolated from Sox2-beta-geo+/–;Mash1–/– embryos, we are performing tissue culture experiments to determine whether growth in G418 results in preferential expansion of a cell population with the characteristics of neural stem cells (one test of this will be to test whether forced expression of Mash1 can rescue the ability of these expanded cells to generate ORNs). These studies will not only aid in our understanding of the basic biology of OE neural stem cells, they will also provide a general strategy for expanding stem cells in the mammalian nervous system.

**Multiple Roles for FGFs in Regulating Olfactory Neurogenesis**

The observation that neuronal progenitors are able to divide and differentiate in serum-free cultures of isolated OE, but neurogenesis ceases in the absence of added factors, led to our investigations to identify signaling molecules that have positive (proneurogenic) effects on OE neurogenesis [10]. In our initial studies, in which we used explant cultures of purified OE to test signaling molecules from several different families, we determined that members of the FGF superfamily are important proneurogenic factors in this system [11]. The FGF gene superfamily consists of 22 related genes that encode polypeptide signaling molecules, many of which exhibit mitogenic properties [42, 43]. Indeed, over the past two decades, numerous studies have shown that FGFs can promote proliferation of many cells of neuroectodermal origin in vitro [44–49]. Our analysis of the actions of FGFs in OE cultures showed that FGFs act to prolong neurogenesis in two ways. First, they act to increase the number of proliferating INPs, by promoting multiple rounds of INP divisions; this action appears to take place in the early G1 phase of the INP cell cycle. Second, FGFs promote proliferation of
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Are FGFs required for neurogenesis and/or proper development of the OE in vivo? To test this idea, we performed sections in situ hybridization of developing and postnatal OE with probes for various Fgfs. We found that a number of different Fgfs are expressed within the OE and that expression of different Fgfs predominates at different developmental ages. Thus, while Fgf8 is expressed within the invaginating epithelium of the olfactory pit during early development, expression of this particular Fgf declines as development proceeds [50, 51; S. Kawau-chi et al., in preparation]. Fgf2, on the other hand, does not appear to be expressed at any significant levels in the developing OE (data not shown) but instead is heavily expressed in the mature OE of adult animals. This is shown in figure 6. In contrast to the expression of Fgf2, which is very strong in adult OE, Fgf8 in adult OE appears to be expressed at a low level, with some higher expression in a few scattered cells (fig. 6A). Interestingly, Fgf8 expression, but not that of Fgf2, can be detected in the basal layer of adult OE, where neuronal progenitors reside (compare the arrow in fig. 6A with Mash1 expression pattern in fig. 6C), whereas Fgf2 expression can be detected in ORN axon bundles (asterisk, fig. 6B). The differential expression of these two FGFs in OE over the lifespan, taken together with their different patterns of expression in adult OE, suggest that FGF8 and FGF2 play very different roles in this tissue. Given that FGF2 lacks a signal sequence for secretion and therefore must be released from expressing cells by another mechanism [42], an intriguing possibility suggests itself: perhaps FGF2 is released by dying ORNs within the OE and thereby plays a role in the regenerative neurogenesis that ORN death is known to stimulate [52, 53]. Future study of neurogenesis during induced regeneration of the OE in animals with an
inactivated Fgf2 gene should help to resolve this question (reviewed in Ornitz and Itoh [42]).

We were particularly interested in Fgf8 as a candidate positive regulator of neurogenesis in developing OE, for several reasons. Among the different Fgf genes, Fgf8 in particular has been reported to be expressed in key signaling centers in early embryos (including the nasal primordium) and has a known role in developmental patterning in limbs and central nervous system [54–56]. Moreover, Fgf8 also has the ability to control cell proliferation when ectopically expressed in the developing nervous system [57]. These observations, coupled with our own preliminary studies showing that recombinant FGF8 has positive effects on OE neuronal progenitor cell proliferation similar to those of FGF2 [58; S. Kawauchi et al., in preparation], support our idea that Fgf8 is likely to play a role in early neurogenesis in the OE. To further investigate this, we examined the expression of Fgf8 in developing OE. Fgf8 expression at E11.5, as well as expression of neuronal lineage markers and the putative stem/progenitor cell markers Sox2 and Raldh3, are shown in figure 6E. Interestingly, the pattern of expression of Fgf8 does not overlap significantly with markers of the ORN neuronal lineage (Mash1, Ngn1 and Ncam).

The critical test of a role for FGF8 in OE neurogenesis is to study the effects of eliminating this signaling molecule’s action in this system. To accomplish this, we are analyzing OE development in mice in which the gene encoding Fgf8 has been inactivated in anterior neural structures, by breeding animals carrying a ‘floxed’ allele of Fgf8 with animals expressing the Cre recombinase in the Foxg1 domain (this approach is necessary because animals with complete inactivation of the Fgf8 gene die at gastrulation, prior to formation of the olfactory placode [59, 60]). These animals are able to complete embryonic development but die as neonates, with large defects in the forebrain and nasal processes, and dramatic reductions in the numbers of neuronal progenitors and neurons in the OE. Thus, our observations indicate that Fgf8 is required for proper OE development [50, 51; S. Kawauchi et al., manuscript in preparation]. The question of whether Fgf8 plays a role in initiation of the neuronal differentiation pathway and/or determination of the olfactory primordium during development, as well as the cell type(s) upon which Fgf8 acts, are currently under investigation [S. Kawauchi et al., manuscript in preparation].

Signaling Molecules of the TGF-ßSuperfamily Exert both Proneurogenic and Antineurogenic Effects in OE

Many studies indicate that bone morphogenetic proteins (BMPs, the largest family of signaling molecules in the TGF-ß superfamily), particularly those of the Dpp (Decapentaplegic) subfamily [61], have antineurogenic actions during development. For example, endogenous BMP4 promotes acquisition of an epidermal fate, at the expense of neural tissue, in developing ectoderm [62, 63], and BMP2 and BMP4 have been shown to inhibit proliferation and/or induce apoptosis of neural progenitor cells in several systems [64–66]. BMPs have antineurogenic actions in the OE as well. In the OE cultures, high – but still physiological – concentrations of BMP2, 4 or 7 inhibit neurogenesis by acting on MASH1-expressing neuronal progenitors: exposure to any of these BMPs causes these progenitors to target pre-existing MASH1 protein for degradation through the proteasome pathway, resulting in apoptosis and termination of the ORN developmental pathway [67].

Studies of the OE have shown that BMPs can exert both positive and negative effects on neurogenesis, however, depending on the concentration and identity of the BMP in question and the identity of the target cell that is acted upon [68]. For example, our studies in vitro have shown that low concentrations of BMP4, but not BMP7, stimulate neurogenesis by promoting survival of newly generated ORNs [68]. Because genetic studies of invertebrate model systems [69, 70] as well as ectopic overexpression studies in mice [71] suggest that cell responses to BMPs may be dictated by the identity of the receptor(s) that are activated, we have begun to explore the role of BMP receptors in the regulation of OE neurogenesis. By testing BMP actions on OE cultures generated from mice in which the BmprIB gene has been inactivated [72], we have found that BmprIB is required for high concentrations of BMP7 to induce degradation of MASH1 in ORN progenitors and inhibit neurogenesis. Interestingly, neither the low-dose proneurogenic effect nor the high-dose antineurogenic effect of BMP4 appears to be affected by the absence of BMPRIB [73; A. Bonnin et al., unpubl. observations]. Both the type IA and type IB BMP receptors are expressed in OE [74, 75; A. Bonnin et al., unpubl. observations], and biochemical studies have shown that BMP4 and BMP7 can bind to and signal through BMPRIB, whereas only BMP4 (but not BMP7) signals through BMPRIA [76]. Taken together with our findings, these observations indicate that BMPRIB acts primarily as a transducer of antineurogenic signals in the OE and suggest that its action is most important for directing the
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Fig. 7. MASH1-expressing neuronal progenitor cell numbers are reduced in the OE of Gdf7–/– mice. Cryostat sections of OE from E14.5 wild-type and Gdf7–/– littermates were processed for MASH1 immunostaining as described in Materials and Methods. BL = Basal lamina; AP = apical surface of epithelium. Scale bar = 20 μm.

response of MASH1-expressing neuronal progenitors. Since BMP7, the preferred ligand for BMPRIB, is also expressed in OE and its underlying stroma [68], our findings also imply that BMPRIB function may be required for mediating antineurogenic signaling by BMP7 in the OE in vivo, a possibility we are currently testing [A. Bonnin et al., unpubl. observations].

A second proneurogenic effect of BMPs, noted in earlier investigations, led to our studies on growth and differentiation factor 7 (GDF7, also known as BMP12). Our earlier studies showed that signals produced by the stroma underlying the OE are required for neurogenesis by isolated OE neuronal progenitor cells grown in vitro [4]. We routinely used this culture system, called the neuronal colony-forming assay, in subsequent studies to demonstrate antineurogenic effects of BMPs [67, 68]. However, when we attempted to use the secreted BMP antagonist noggin [77] to abrogate the antineurogenic action of BMPs in neuronal colony-forming assays, we found that we were unable to perform such experiments because noggin alone blocked all neurogenesis in such cultures [68]. Further tests revealed that OE stromal cells secrete noggin-binding factor(s) that stimulate OE neurogenesis, and this factor or factors was presumed to be the target of noggin’s antineurogenic action in the colony-forming assays [68].

At least one biological activity of GDF7, part of the BMP family of the TGF-β group, had previously been shown to be antagonized by noggin [78]. Because genetic studies also showed that Gdf7 is required for the development of a subclass of spinal interneurons in mice [78], we considered it a candidate proneurogenic, noggin-antagonizable BMP for the OE system and examined its expression in the nasal mucosa by in situ hybridization. These studies showed that Gdf7 is expressed by stromal cells underlying the OE, but not in the OE proper [79; C. Crocker, J. Kim and A.L. Calof, unpubl. results]. Since recombinant GDF7 also promotes neurogenesis in OE cultures, and the target of its proneurogenic effect appears to be the MASH1-expressing neuronal progenitor [79, 80; C. Crocker et al., manuscript in preparation], we were prompted to see if loss of Gdf7 function resulted in deficits in these progenitors in the OE in vivo as well. For these experiments, we used an antibody to MASH1 to examine the OE of Gdf7–/– embryos at E14.5, the age at which tissue is normally taken for culture [81]. The results are shown in figure 7. The number of MASH1-immunopositive cells is reduced by almost half [48%; wild type = 138.9 ± 16.5 (SEM) MASH1+ cells/mm; Gdf7–/– = 66.7 ± 14.1 (SEM) MASH1+ cells/mm; >6 mm counted per animal, with 4 animals of each genotype assessed] in the OE of Gdf7–/– embryos, indicating that Gdf7 acts as a positive regulator of developmental neurogenesis in the OE in vivo, as well as in vitro [C. Crocker et al., manuscript in preparation]. Since proliferation of MASH1-expressing neuronal progenitors is known to be dramatically upregulated during the early stages of regenerative neurogenesis in adult OE [3], it will be interesting to use Gdf7–/– animals (which survive to early adulthood [78; C. Crocker et al., unpubl. observations]) to determine if GDF7 is required for this response as well.

Feedback Inhibition of Neurogenesis and Maintenance of the Regenerative Response

The OE – unlike nearly all other regions of the vertebrate nervous system – can regenerate its neurons even in adult animals; this has made it possible to learn that neurogenesis in the OE is a highly regulated process that maintains the number of ORNs at a particular level.
Fig. 8. **A** Expression of *Gdf11* and *Fst* in adult OE. Horizontal cryostat sections through the OE of a 6-week-old CD-1 mouse were hybridized with probes to *Gdf11*, *Fst* and *Gdf8* as described in Materials and Methods. BL = Basal lamina; SUS = sustentacular cells. Scale bar = 20 μm. **B** Summary of molecular signals in OE neurogenesis. During primary OE neurogenesis (E9.5–E12.5), FGF and other factors act to increase stem cell number and establish the different cell types in the ORN lineage. After E12.5, the OE neuronal lineage is established (see fig. 1A). FGFs and TGF-ßs converge on different cell types in the OE neurogenic lineage to achieve and maintain proper neuron number.

Thus, in normal animals, in which ORNs are constantly dying in low numbers (due to disease or environmental insult), a low level of production of new ORNs is constantly replacing them. If surgical or chemical manipulations are used to eliminate large numbers of ORNs abruptly, the production of new neurons is markedly upregulated until the original state of the OE is restored (reviewed in Murray and Calof [1] and Calof et al. [53]). The temporal relationship of ORN death, cell degeneration and progenitor cell proliferation suggests that neuronal progenitors ‘read’ the number of neurons in their environment and regulate the production of new neurons accordingly. This has led us to hypothesize that neuron number in the OE is regulated by a negative feedback process in which ORNs produce a signal that inhibits proliferation of their own progenitors. Recently, we demonstrated that growth and differentiation factor 11 (GDF11, a member of a small subgroup of activin-like TGF-ß [82]) has the characteristics of such a feedback-inhibitory signal [9]. *Gdf11* is expressed by both INPs and ORNs in the OE, as are its transmembrane receptors and its secreted antagonist follistatin. Recombinant GDF11 inhibits OE neurogenesis in vitro, by reversibly arresting INP divisions. There are several interesting aspects of this effect: (1) it
predominates over the proliferative effect of FGFs on INPs [11]; (2) it is accompanied by induction of the cyclin-dependent kinase inhibitor p27Kip1 in neuronal progenitors, and (3) it is abrogated by addition of follistatin to the cultures [9]. Finally, and most importantly, Gdf11 knockout mice show increased neurogenesis, whereas Fst–/– mice show decreased neurogenesis, in the OE during embryonic development [9]. These findings demonstrated that GDF11 is a critical endogenous negative regulator of developmental neurogenesis in the OE, and suggested in addition that this signaling molecule might interact with proneurogenic factors such as FGFs to regulate the progress of neuronal progenitors and/or stem cells through the ORN differentiation pathway (fig. 8).

Because Gdf11 and Fst null mice die at birth [9, 83, 84], a role for GDF11 in autoregulation of neurogenesis during OE regeneration has not yet been explored using a genetic approach. However, such regeneration recapitulates many of the events of development [3, 85] and therefore may depend on the same regulatory molecules. Our preliminary experiments using in situ hybridization indicate that Gdf11 and Fst continue to be expressed in the OE through adult life. This is shown in figure 8. Interestingly, Gdf11 appears to be most highly expressed in the basal third of the OE, suggesting that it is expressed primarily by neuronal progenitor cells and a few layers of immature ORNs immediately above them. Fst, in contrast, appears to be expressed by most, if not all, ORNs, but not in the apical sustentacular cell layer or in the most basal cell layers containing neuronal progenitors. Understanding the significance of these patterns of expression in terms of the regenerative capacity of the OE is currently a major area of investigation for our laboratory.

What is the potential significance of GDF11 for regeneration in the OE? The actions of GDF11 appear to be directed mainly toward INPs, the most abundant progenitor cell type in the ORN lineage and the final stage of proliferation in the OE. Finally, our studies demonstrate that the TGF-βs and their antagonists appear to be key players in the feedback loops that regulate the size of progenitor cell pools and neuron number during developmental neurogenesis; the possibility that such feedback mechanisms regulate neuronal regeneration as well remains to be investigated. Finally, an unforeseen positive outcome of the process of determining the actions of these signaling molecules has been the identification of new molecular markers for neural stem cells, as well as transgenic model systems which should facilitate the isolation and study of neural stem cells in development and regeneration.

### Methods

#### Animals

Mice were naturally mated, with the morning of appearance of a vaginal plug designated as embryonic day 0.5 (E0.5). Mash1+/- mice [6] and BmprIIB+/– mice [72] were maintained on an outbred CD-1 (Charles River Laboratories) background, where OE phenotypes are penetrant. Sox2beta-geo [80] and Foxg1-Cre [60] and Fgf8floxp mice [59] were maintained on a Swiss Webster (Simonsen) background. GDF7+/– mice [78] were maintained for ≥ 6 generations on a C57BL/6J background (Jackson).

#### In situ Hybridization and Probes

Embryos were fixed 2–4 h at room temperature (up to E14.5) or overnight at 4°C (E17.5–adult), in 4% paraformaldehyde/phosphate-buffered saline (PBS). Embryos were cryoprotected in 30% sucrose/PBS and embedded in tissue freezing medium (Triangle Biochemicals Durham, N.C., USA), then sectioned at 20 μm thickness on a cryostat onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa., USA). Sections were stored at –20°C until used.

In situ hybridization was performed as described previously [8]. The signal was visualized using 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride as substrate (final concentration: 175 μg/ml of bromo-4-chloro-3-indolyl-phosphate and 350 μg/ml nitroblue tetrazolium; reagents were from Roche Molecular Biochemicals, Indianapolis, Ind., USA). Sections were incubated at...
room temperature or 4 °C until the signal was clearly visualized. Slides were dehydrated rapidly through xylens and mounted in Pro-Texx (Lerner Laboratories, Pittsburgh, Pa., USA).

Ribonucleotide probes were synthesized with either T7, T3 or SP6 RNA polymerase (Fisher Scientific) using digoxigenin-UTP as label (Roche). Mash1, Ngn1, Ncam and Steel probes were described previously [8]. Raldh1, Raldh2 and Raldh3 probes contained the entire coding region of each gene (gifts of Dr. F. Grün, UC Irvine): Raldh1 = bp 31–1536 of Genbank No. BC054386; Raldh2 = bp 57–1556 of No. NM009022; Raldh3 = bp 21–1535 of No. AF15235. The 721-bp probe for mouse Fg8 covered the entire coding region (Genbank No. MMU18673) and was from Dr. I. Mason (King’s College, London, UK). 1.3 kb of mouse Fgf2 was obtained from IMAGE clone No. 1447251, 748 bp of mouse Sox2 (bp 1281–2029 of Genbank No. X94127) was the gift of Dr. E.J. Robertson (Harvard University, Cambridge, Mass., USA). Probes for mouse Gdf8, Gdf11, Alk5, ActRIIb and Fst were described previously [9].

β-Galactosidase Histochemistry and MASH1 Immunostaining

E12.5 Sox2+/neo− hemizygous embryos were fixed and sectioned for histochemical detection of β-galactosidase activity as previously described [8]. Sections were stained for 30 min in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Inalco Spa, Milano, Italy). 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.1% Triton X-100, 0.01% deoxycholate, in PBS at 37 °C, and then dehydrated and mounted in Pro-Texx. Anti-MASH1 immunostaining was performed on E14.5 CD-1 mouse embryo heads that were fixed by immersion for 3 h at room temperature in 10% formalin/PBS/5% sucrose, then cryoprotected and sectioned at 12 μm in the horizontal plane. Sections were stained using hybridoma supernatant from A42B7 MASH1 hybridoma [88] as described previously [3].

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