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

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Key Words

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

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 neuro-

genesis 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- β s 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.

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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,

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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.

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 differ-

ent 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. 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-

genitor cells within the OE. To characterize the cells that express RALDHs, we have performed preliminary tests using a fluorescent substrate for RALDHs [Bodipy aminoacetaldehyde (BAAA)]. RALDHs oxidize BAAA in the cytosol and convert it to Bodipy aminoacetate (BAA), 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 BAAA 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 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* 3' 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* 3' 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 *SoxB1* 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).

Fig. 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 μ m.

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].

Fig. 5. *Sox2-beta-geo* expression in the nervous system at E12. Thirty-micrometer-thick horizontal cryosection of *Sox2-beta-geo* hemizygous embryo head, stained with X-gal as described in the Methods. **A** Low-power section through the head. X-gal staining is most evident in the neural retina (NR), OE and cerebellar primordium (CPri), as well as the ventricular zone of the diencephalon (no label); the trigeminal ganglion (VG) is lightly stained. **B** Higher-power image of OE from **A**, demonstrating abundant X-gal staining in basal and apical cell layers. NC = Nasal cavity. Scale bar = 50 μ m. **C** High-power view of the region boxed in **B**, showing heavily labeled cells in the apical OE (AL), as well as in patches in the basal layer, adjacent to the basal lamina (BL). Scale bar = 25 μ m.

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 understand-

ing 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

Fig. 6. A–D In situ hybridization on sections (20 μ m) of paraformaldehyde-fixed OE of young adult (3 weeks of age) CD-1 mice. **A** *Fgf8* (developed overnight at room temperature). The arrow indicates *Fgf8*-expressing cells in the basal layer of the OE. **B** *Fgf2* (developed 4 h at room temperature), section adjacent to that shown in **A**. The asterisk indicates an *Fgf2*-expressing ORN axon bundle running in the stroma subjacent to the OE. **C** *Mash1* (developed 5 h at room temperature). **D** *Fgf2* sense control (development time as for **B**).

Sus = Sustentacular cell layer; BL = basal lamina. Scale bar = 25 μ m. **E** Expression of *Fgf8* mRNA and other stem and neuronal cell markers in the OE at E11.5. *Fgf8* is expressed in the OE at the rim of the invaginating nasal cavity. FB = Forebrain; NC = nasal cavity; MNP = medial nasal process; LNP = lateral nasal process. *Sox2* and *Raldh3* are markers of progenitor/putative stem cells. The region of expression of neuronal lineage markers *Mash1*, *Ngn1* and *Ncam* does not overlap with that of *Fgf8*. Scale bar = 100 μ m.

rare progenitor cells – putative ORN stem cells – in a small fraction of OE explants [11]. These studies also provided the first evidence that INPs behave as transit amplifying progenitors in the ORN lineage and that stem cells could be identified in long-term cultures of OE explants.

Are FGFs required for neurogenesis and/or proper development of the OE in vivo? To test this idea, we performed section 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. Kawachi 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- β Superfamily 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- β 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 *Bmpr1B* gene has been inactivated [72], we have found that *Bmpr1B* 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 BMPR1B [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 BMPR1B, whereas only BMP4 (but not BMP7) signals through BMPRIA [76]. Taken together with our findings, these observations indicate that BMPR1B acts primarily as a transducer of antineurogenic signals in the OE and suggest that its action is most important for directing the

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 μ m.

response of MASH1-expressing neuronal progenitors. Since BMP7, the preferred ligand for BMPRII, is also expressed in OE and its underlying stroma [68], our findings also imply that BMPRII 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.

predominates over the proliferative effect of FGFs on INPs [11]; (2) it is accompanied by induction of the cyclin-dependent kinase inhibitor p27^{Kip1} 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 proliferating progenitors in the ORN differentiation pathway [9]. INPs function as transit amplifying cells in the OE, in vitro and in vivo [3, 11] – this is most notable in paradigms in which surgical ablation of the olfactory bulb induces a massive wave of ORN apoptosis [52], resulting in a surge of INP proliferation that is maintained until new ORNs are generated (reviewed in Calof et al. [53]). Since GDF11 arrests INP proliferation but does not kill INPs, it may be important for maintaining these cells in a state of readiness to respond rapidly in order to replace dying ORNs, the function of transit-amplifying cells in many regenerating tissues [86, 87].

Summary and Conclusions

Taking a candidate approach to identifying signaling molecules that regulate neurogenesis in the OE model system, we have identified multiple members of the FGF and TGF- β superfamilies that have important and specific effects on each of the three defined stem/progenitor cell stages in the ORN lineage. Our studies indicate that different FGFs and TGF- β s have distinct positive (proneurogenic) and negative (antineurogenic) actions on OE progenitors at different stages of differentiation. Interestingly, our experiments also indicate the possibility that opposing signals can converge on cells at a single developmental stage to regulate progress through the ORN differentiation pathway. Finally, our studies demonstrate that the TGF- β s and their antagonists appear to be key players in 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 *Bmpr1B*^{+/-} mice [72] were maintained on an outbred CD-1 (Charles River Laboratories) background, where OE phenotypes are fully penetrant. *Sox2*^{beta-geo} hemizygotes [40] were on a mixed 129/sv/CD-1 background, and *Foxg1-Cre* [60] and *Fgf8*^{d2, 3} and *Fgf8*^{lox} mice [59] were maintained on a Swiss Webster (Simonsen) background. *GDF7*^{+/-} mice [78] were maintained for ≥ 6 generations on a C57/BL6J 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 Bio-medical Sciences Durham, N.C., USA), then sectioned at 20 μ m thickness on a cryostat onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa., USA). Sections were stored at -80°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 μ g/ml of bromochloroindolylphosphate and 350 μ g/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 xylenes 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 21–1559 of No. AF15235. The 721-bp probe for mouse *Fgf8* 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^{beta-geo}* 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 μ m in the horizontal plane. Sections were stained using hybridoma supernatant from A42B7 MASH1 hybridoma [88] as described previously [3].

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References

- Murray RC, Calof AL: Neuronal regeneration: Lessons from the olfactory system. *Semin Cell Dev Biol* 1999;10:421–431.
- Calof AL, Bonnin A, Crocker C, Kawauchi S, Murray RC, Shou J, Wu HH: Progenitor cells of the olfactory receptor neuron lineage. *Microsc Res Tech* 2002;58:176–188.
- Gordon MK, Mumm JS, Davis RA, Holcomb JD, Calof AL: Dynamics of MASH1 expression in vitro and in vivo suggest a non-stem cell site of MASH1 action in the olfactory receptor neuron lineage. *Mol Cell Neurosci* 1995;6:363–379.
- Mumm JS, Shou J, Calof AL: Colony-forming progenitors from mouse olfactory epithelium: Evidence for feedback regulation of neuron production. *Proc Natl Acad Sci USA* 1996;93:11167–11172.
- Calof AL, Mumm JS, Rim PC, Shou J: The neuronal stem cell of the olfactory epithelium. *J Neurobiol* 1998;36:190–205.
- Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL: Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. *Cell* 1993;75:463–476.
- Cau E, Gradwohl G, Fode C, Guillemot F: Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* 1997;124:1611–1621.
- Murray RC, Navi D, Fesenko J, Lander AD, Calof AL: Widespread defects in the primary olfactory pathway caused by loss of Mash1 function. *J Neurosci* 2003;23:1769–1780.
- Wu HH, Ivkovic S, Murray RC, Jaramillo S, Lyons KM, Johnson JE, Calof AL: Autoregulation of neurogenesis by GDF11. *Neuron* 2003;37:197–207.
- Calof AL, Chikaraishi DM: Analysis of neurogenesis in a mammalian neuroepithelium: Proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron* 1989;3:115–127.
- DeHamer MK, Guevara JL, Hannon K, Olwin BB, Calof AL: Genesis of olfactory receptor neurons in vitro: Regulation of progenitor cell divisions by fibroblast growth factors. *Neuron* 1994;13:1083–1097.
- Farbman AI: *Cell Biology of Olfaction*. Cambridge, Cambridge University Press, 1992.
- Morriss-Kay GM, Ward SJ: Retinoids and mammalian development. *Int Rev Cytol* 1999;188:73–131.
- Dickman ED, Thaller C, Smith SM: Temporally regulated retinoic acid depletion produces specific neural crest, ocular and nervous system defects. *Development* 1997;124:3111–3121.
- Schneider RA, Hu D, Rubenstein JL, Maden M, Helms JA: Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. *Development* 2001;128:2755–2767.
- Smith SM, Dickman ED, Power SC, Lancman J: Retinoids and their receptors in vertebrate embryogenesis. *J Nutr* 1998;128:467S–470S.
- Maden M, Gale E, Kostetskii I, Zile M: Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr Biol* 1996;6:417–426.
- Callaerts P, Halder G, Gehring WJ: PAX-6 in development and evolution. *Annu Rev Neurosci* 1997;20:483–532.
- Walther C, Gruss P: Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* 1991;113:1435–1449.
- Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND, van Heyningen V: Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* 1991;354:522–525.
- Hogan BL, Hirst EM, Horsburgh G, Hetherington CM: Small eye (Sey): A mouse model for the genetic analysis of craniofacial abnormalities. *Development* 1988;103:115–119.
- Hogan BL, Horsburgh G, Cohen J, Hetherington CM, Fisher G, Lyon MF: Small eyes (Sey): A homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J Embryol Exp Morphol* 1986;97:95–110.
- Fujiwara M, Uchida T, Osumi-Yamashita N, Eto K: Uchida rat (rSey): A new mutant rat with craniofacial abnormalities resembling those of the mouse Sey mutant. *Differentiation* 1994;57:31–38.
- Grindley JC, Davidson DR, Hill RE: The role of Pax-6 in eye and nasal development. *Development* 1995;121:1433–1442.
- Jimenez D, Garcia C, de Castro F, Chedotal A, Sotelo C, de Carlos JA, Valverde F, Lopez-Mascaraque L: Evidence for intrinsic development of olfactory structures in Pax-6 mutant mice. *J Comp Neurol* 2000;428:511–526.

- 26 Anchan RM, Drake DP, Haines CF, Gerwe EA, LaMantia AS: Disruption of local retinoid-mediated gene expression accompanies abnormal development in the mammalian olfactory pathway (published erratum appears in J Comp Neurol 1997;384:321). J Comp Neurol 1997;379:171–184.
- 27 Suzuki R, Shintani T, Sakuta H, Kato A, Ohkawara T, Osumi N, Noda M: Identification of RALDH-3, a novel retinaldehyde dehydrogenase, expressed in the ventral region of the retina. Mech Dev 2000;98:37–50.
- 28 McCaffery P, Drager UC: Regulation of retinoic acid signaling in the embryonic nervous system: A master differentiation factor. Cytokine Growth Factor Rev 2000;11:233–249.
- 29 Lin M, Zhang M, Abraham M, Smith SM, Napoli JL: Mouse retinal dehydrogenase 4 (RALDH4), molecular cloning, cellular expression, and activity in 9-*cis*-retinoic acid biosynthesis in intact cells. J Biol Chem 2003;278:9856–9861.
- 30 Grun F, Hirose Y, Kawauchi S, Ogura T, Umesono K: Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. J Biol Chem 2000;275:41210–41218.
- 31 Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Ludeman SM, Smith C: Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 1999;96:9118–9123.
- 32 Dupe V, Matt N, Garnier JM, Chambon P, Mark M, Ghyselinck NB: A newborn lethal defect due to inactivation of retinaldehyde dehydrogenase type 3 is prevented by maternal retinoic acid treatment. Proc Natl Acad Sci USA 2003;100:14036–14041.
- 33 Wood HB, Episkopou V: Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech Dev 1999;86:197–201.
- 34 Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ, Sharpe PT: Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development. Mech Dev 1995;49:23–36.
- 35 Pevny LH, Sockanathan S, Placzek M, Lovell-Badge R: A role for SOX1 in neural determination. Development 1998;125:1967–1978.
- 36 Bylund M, Andersson E, Novitsch BG, Muhr J: Vertebrate neurogenesis is counteracted by Sox1–3 activity. Nat Neurosci 2003;6:1162–1168.
- 37 Graham V, Khudyakov J, Ellis P, Pevny L: SOX2 functions to maintain neural progenitor identity. Neuron 2003;39:749–765.
- 38 Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R: Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 2003;17:126–140.
- 39 Li M, Pevny L, Lovell-Badge R, Smith A: Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr Biol 1998;8:971–974.
- 40 Zappone MV, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK: Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 2000;127:2367–2382.
- 41 Smart IH: Location and orientation of mitotic figures in the developing mouse olfactory epithelium. J Anat 1971;109:243–251.
- 42 Ornitz DM, Itoh N: Fibroblast growth factors. Genome Biol 2001;2:3005.1–3005.12.
- 43 Ford-Perriss M, Abud H, Murphy M: Fibroblast growth factors in the developing central nervous system. Clin Exp Pharmacol Physiol 2001;28:493–503.
- 44 Hughes RA, Sendtner M, Goldfarb M, Lindholm D, Thoenen H: Evidence that fibroblast growth factor 5 is a major muscle-derived survival factor for cultured spinal motoneurons. Neuron 1993;10:369–377.
- 45 Hajihosseini MK, Dickson C: A subset of fibroblast growth factors (Fgfs) promote survival, but Fgf-8b specifically promotes astroglial differentiation of rat cortical precursor cells. Mol Cell Neurosci 1999;14:468–485.
- 46 Ghosh A, Greenberg ME: Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 1995;15:89–103.
- 47 Temple S, Qian X: bFGF, neurotrophins, and the control of cortical neurogenesis. Neuron 1995;15:249–252.
- 48 Eckenstein FP: Fibroblast growth factors in the nervous system. J Neurobiol 1994;25:1467–1480.
- 49 Mason IJ: The ins and outs of fibroblast growth factors. Cell 1994;78:547–552.
- 50 Kawauchi S, Shou J, Calof AL: Requirement for Fgf8 in olfactory neurogenesis (SDB 2002 Abstr). Dev Biol 2002;247:449.
- 51 Kawauchi S, Shou J, Santos R, Calof AL: Role of Fgf8 in regulating olfactory neurogenesis. Soc Neurol Abstr 2003;29:349.
- 52 Holcomb JD, Mumm JS, Calof AL: Apoptosis in the neuronal lineage of the mouse olfactory epithelium: Regulation in vivo and in vitro. Dev Biol 1995;172:307–323.
- 53 Calof AL, Hagiwara N, Holcomb JD, Mumm JS, Shou J: Neurogenesis and cell death in olfactory epithelium. J Neurobiol 1996;30:67–81.
- 54 Crossley PH, Martin GR: The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 1995;121:439–451.
- 55 Mahmood R, Bresnick J, Hornbruch A, Mahony C, Morton N, Colquhoun K, Martin P, Lumsden A, Dickson C, Mason I: A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. Curr Biol 1995;5:797–806.
- 56 Martinez S, Crossley PH, Cobos I, Rubenstein JL, Martin GR: FGF8 induces formation of an ectopic isthmus organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development 1999;126:1189–1200.
- 57 Lee SM, Danielian PS, Fritzsche B, McMahon AP: Evidence that FGF8 signalling from the midbrain-hindbrain junction regulates growth and polarity in the developing midbrain. Development 1997;124:959–969.
- 58 Calof AL, Iannuzzi P, Rim P, MacArthur C, Mason I, Shou J: Potential autocrine role for FGF8 in regulating neurogenesis in mammalian olfactory epithelium. Soc Neurol Abstr 1997;23:298.
- 59 Meyers EN, Lewandoski M, Martin GR: An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nat Genet 1998;18:136–141.
- 60 Hebert JM, McConnell SK: Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. Dev Biol 2000;222:296–306.
- 61 Newfied SJ, Wisotzky RG, Kumar S: Molecular evolution of a developmental pathway: Phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics 1999;152:783–795.
- 62 Hawley SHB, Wunnenberg-Stapleton K, Hashimoto C, Laurent MN, Watabe T, Blumberg BW, Cho KKY: Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. Genes Dev 1995;9:2923–2935.
- 63 Wilson PA, Hemmati-Brivanlou A: Induction of epidermis and inhibition of neural fate by Bmp-4. Nature 1995;376:331–336.
- 64 Furuta Y, Piston DW, Hogan BL: Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 1997;124:2203–2212.
- 65 Golden JA, Braciclovic A, McFadden KA, Beesley JS, Rubenstein JL, Grinspan JB: Ectopic bone morphogenetic proteins 5 and 4 in the chicken forebrain lead to cyclopia and holoprosencephaly. Proc Natl Acad Sci USA 1999;96:2439–2444.
- 66 Graham A, Francis-West P, Brickell P, Lumsden A: The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature 1994;372:684–686.
- 67 Shou J, Rim PC, Calof AL: BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. Nat Neurosci 1999;2:339–345.
- 68 Shou J, Murray RC, Rim PC, Calof AL: Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. Development 2000;127:5403–5413.
- 69 Krishna S, Maduzia LL, Padgett RW: Specificity of TGFbeta signaling is conferred by distinct type I receptors and their associated SMAD proteins in *Caenorhabditis elegans*. Development 1999;126:251–260.

- 70 Nguyen M, Park S, Marques G, Arora K: Interpretation of a BMP activity gradient in *Drosophila* embryos depends on synergistic signaling by two type I receptors, SAX and TKV. *Cell* 1998;95:495–506.
- 71 Panchision DM, Pickel JM, Studer L, Lee SH, Turner PA, Hazel TG, McKay RD: Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev* 2001;15:2094–2110.
- 72 Yi SE, Daluiski A, Pederson R, Rosen V, Lyons KM: The type I BMP receptor BMPRII is required for chondrogenesis in the mouse limb. *Development* 2000;127:621–630.
- 73 Bonnin A, Chern P, Yi S, Lyons K, Calof A: The type I bone morphogenetic protein receptor BMPRII is required for the antineurogenic effect of BMP7 in the olfactory epithelium. *Soc Neurol Abstr* 2000;26:1087.
- 74 Dewulf N, Verschuere K, Lonnoy O, Moren A, Grimsby S, Vande Spiegle K, Miyazono K, Huylebroeck D, Ten Dijke P: Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology* 1995;136:2652–2663.
- 75 Zhang D, Mehler MF, Song Q, Kessler JA: Development of bone morphogenetic protein receptors in the nervous system and possible roles in regulating *trkC* expression. *J Neurosci* 1998;18:3314–3326.
- 76 ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K: Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J Biol Chem* 1994;269:16985–16988.
- 77 Zimmerman LB, Jesus-Escobar JMd, Harland RM: The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 1996;86:599–606.
- 78 Lee KJ, Mendelsohn M, Jessell TM: Neuronal patterning by BMPs: A requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev* 1998;12:3394–3407.
- 79 Crocker CE, Wu H-H, Bonnin A, Calof AL: Actions of bone morphogenetic protein 12 (GDF7) on neuronal progenitors of mouse olfactory epithelium. *Soc Neurol Abstr* 2001;27:934.
- 80 Crocker CE, Chern PL, Murray RC, Calof AL: Role of bone morphogenetic protein 12 (GDF7) in regulating neurogenesis in the mouse olfactory epithelium. *Soc Neurol Abstr* 2000;26:1343.
- 81 Calof AL, Mumm JS, Rim PC, Shou J: In vitro analysis of neuronal progenitor cells from mouse olfactory epithelium; in Haynes L (ed): *The Neuron in Tissue Culture*. Chichester, Wiley, 1999, pp 23–44.
- 82 Nakashima M, Toyono T, Akamine A, Joyner A: Expression of growth/differentiation factor 11, a new member of the BMP/TGF β superfamily during mouse embryogenesis. *Mech Dev* 1999;80:185–189.
- 83 McPherron AC, Lawler AM, Lee SJ: Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nat Genet* 1999;22:260–264.
- 84 Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A: Multiple defects and perinatal death in mice deficient in follistatin. *Nature* 1995;374:360–363.
- 85 Calof AL, Holcomb JD, Mumm JS, Hagiwara N, Tran P, Smith KM, Shelton D: Factors affecting neuronal birth and death in the mammalian olfactory epithelium. *Ciba Found Symp* 1996;196:188–205.
- 86 Hall PA, Watt FM: Stem cells: The generation and maintenance of cellular diversity. *Development* 1989;106:619–633.
- 87 Potten CS, Loeffler M: Stem cells: Attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 1990;110:1001–1020.
- 88 Lo LC, Johnson JE, Wuenschell CW, Saito T, Anderson DJ: Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. *Genes Dev* 1991;5:1524–1537.