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Chapter 3

Negative Regulation of Endogenous Stem Cells in Sensory Neuroepithelia: Implications for Neurotherapeutics

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Abstract Stem cell therapies to treat central nervous system (CNS) injuries and diseases face many obstacles, one of which is the fact that the adult CNS often presents an environment hostile to the development and differentiation of neural stem and progenitor cells. Close examination of two regions of the nervous system – the olfactory epithelium (OE), which regenerates, and the neural retina, which does not – have helped identify endogenous signals, made by differentiated neurons, which act to inhibit neurogenesis by stem/progenitor cells within these tissues. In this chapter, we provide background information on these systems and their neurogenic signaling systems, with the goal of providing insight into how manipulation of endogenous signaling molecules may enhance the efficacy of stem cell neurotherapeutics.

Keywords BMP, FGF, follistatin, GDF11, proneural genes, regeneration, Sox2

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3.1 Introduction

Stem cells are increasingly viewed as viable sources of treatment for injured or diseased nervous system tissues, in which the mammalian capacity for regeneration of damaged tissue is severely limited. Great efforts have been made to understand the molecular signals regulating the growth and differentiation of neural stem cells during normal development in many model systems. Understanding the molecular signals that regulate endogenous stem cell populations will provide information that should eventually permit us to harness these signals and stimulate growth and regeneration of neural tissues from endogenous stem cell pools. In addition, such information will permit us to utilize appropriate molecular tools for production of specialized neural cell types *in vitro* for use in transplantation-based therapies.

3.2 Olfactory Epithelium as a Model System for Understanding Molecular Regulation of Endogenous Neural Stem Cells

The adult mammalian central nervous system (CNS), relative to other tissues, possesses a severely limited cohort of stem cells. Following development, basal stem cell activity is low, suggesting that stem cells are under tight negative regulation. This regulation, which likely keeps stem cells in a “locked” or dormant state, is in part responsible for the poor regeneration seen in injured or diseased CNS. Currently, only three mammalian CNS stem cell niches are known to persist into adulthood: the subventricular zone (SVZ) of the lateral ventricle, the subgranular layer (SGL) of the dentate gyrus, and the olfactory epithelium (OE) [reviewed in 1, 2].

The OE is unique amongst mammalian neurogenic tissues: it continually generates neurons throughout life, making it an attractive model system for studying not only neurogenesis, but neural regeneration as well. OE neurogenesis is tightly regulated to allow for regeneration when many or most neurons are lost, but also to prevent abnormal overgrowth. It is possible that a similar regulatory mechanism may repress neurogenesis within non-regenerative neural tissues. An understanding of the molecular signals that permit ongoing OE neurogenesis and regulate regeneration should provide insight into how these same signals could be harnessed to promote regeneration of other brain regions following disease or injury.

3.2.1 Structure and Development of the Olfactory Epithelium

The posterodorsal region of the nasal cavity in mammals (Fig. 3.1A) is lined with an olfactory mucosa consisting of the olfactory epithelium (OE) and its underlying lamina propria [3–5]. OE development begins around gestational day 9 (E9) in mice, at which time bilateral thickenings of the surface ectoderm, called the olfactory pla-

codes (OPs), are first evident. The OP invaginates to form the olfactory pit, and over time the epithelium thickens and comes to consist of three major cellular compartments: (1) apical, (2) basal, and (3) intermediate or middle (Fig. 3.1B). The apical layer is adjacent to the nasal cavity, and is comprised of a single layer of supporting or sustentacular (SUS) cells. These cells extend their endfeet to the basal lamina (BL) and, like glia of the CNS, provide architectural support to growing neurons [6, 7]. Atop the BL lies the basal compartment, which contains a single layer of horizontal basal cells (HBCs) and one to two layers of globose basal cells (GBCs). HBCs are situated closest to the BL and express keratin intermediate filaments [8]. Although HBCs do not appear to be part of the OE neuronal lineage during development, severe induced damage to the OE has been shown to stimulate this population to repopulate the OE, at least partially [9–11]. GBCs lie directly above the HBCs. GBCs are actually a mixed cell population, which has been shown to contain the stem/progenitor cell types that give rise to olfactory receptor neurons (ORNs) in vivo [12–15]. Between sustentacular and progenitor cells sit four to five layers of ORNs, the sensory neurons of the OE. The axons of ORNs project subjacent to the epithelium into the lamina propria, through the cribriform plate of the ethmoid bone, directly into the CNS, where they synapse on neurons of the main olfactory bulb (Fig. 3.1A). An additional olfactory sensory epithelium, the vomeronasal organ (VNO), lies within the septum ventral to main OE (Fig. 3.1A). The VNO detects pheromonal chemical signals that influence mating and social behavior. Afferent axons of vomeronasal neurons connect to the accessory olfactory bulb.

Studies by us and others have shown that ORNs are generated via a lineage consisting of three distinct proliferating cell types identified by specific markers (Fig. 3.1C). (1) The *neural stem cell*, which expresses *Sox2*, a SRY-family transcription factor expressed by many stem cells including embryonic stem cells and many neuroepithelial cells, is the first cell in the lineage [1, 16–21]. *Sox2*-expressing stem cells give rise to (2) *committed progenitor cells* that express *Mash1* (*Ascl1*), a proneural gene that encodes a basic helix-loop-helix transcription factor required for ORN development [22–24]. *Mash1*-expressing neuronal progenitors give rise to (3) *immediate neuronal precursors* (INPs), which expresses the proneural gene *Neurogenin1* (*Ngn1*) and give rise to daughter cells that undergo terminal differentiation into ORNs (Fig. 3.1C) [1, 8]. ORNs, which are the odor signal-transducing neurons of the OE, reside within the middle compartment, sandwiched between the apical and basal cell layers. ORNs extend cilia into the nasal cavity and axons into the CNS (Fig. 3.1). All postmitotic ORNs can be identified by their expression of the neural cell adhesion molecule, NCAM [8].

3.2.2 *FGF8* Expression Defines Primordial Neural Stem Cells During Early OE Development

These three cell types are evident from the earliest stages of OE development [22, 25]. Expression patterns from in situ hybridization studies indicate that, at the

Fig. 3.1 Schematic drawings of olfactory structures and relevant developmental cell types. (A) Sagittal view of the nasal cavity and rostral forebrain of a mouse. The olfactory epithelium (OE) lines the nasal cavity and contains olfactory receptor neurons (ORNs), which project through the cribriform plate to glomeruli in the main olfactory bulb (MOB) within the central nervous system. Pheromone-detecting vomeronasal neurons (VNN) of the vomeronasal organ (VNO) project to the accessory olfactory bulb (AOB). (B) Diagram of the OE showing relative positions of cell types. From apical to basal, these include sustentacular cells (SUS; green), ORNs, and globose and horizontal basal cells (GBC; HBC). The GBCs consist of three types of neuronal progenitors: *Sox2*-expressing neuronal stem cells, *Mash1*-expressing transit amplifying progenitors, and *Ngn1*-expressing INPs. (C) Schematic of the neuronal differentiation pathway of OE neuronal progenitor cells. *Sox2*-expressing neuronal stem cells give rise to transit amplifying progenitors expressing *Mash1*, which produce *Ngn1*-expressing immediate neuronal precursors (INPs). INP division produces daughter cells that differentiate into immature ORNs, identified by NCAM immunoreactivity. Immature ORNs eventually mature and express NCAM and olfactory marker protein (OMP) (See Color Plates)

early olfactory pit stage, stem and progenitor cell types of the OE are arranged in a concentric, outside-in pattern that reflects the developmental stage of each cell type in the OE lineage (Fig. 3.2). At this stage of development, the outer margin of the invaginating nasal pit (NP) is also marked by expression of an important regulatory signaling molecule of the fibroblast growth factor (FGF) superfamily, FGF8 (Fig. 3.2A, B) [25]. Closest to the *Fgf8*-expressing cells at the inner rim of the invaginating olfactory pit are cells expressing *Mash1*, the earliest committed neuronal progenitors of the OE. Further in toward the center of the pit are the *Ngn1*-expressing INPs; and at the center are *Ncam1*-expressing ORNs (Fig. 3.2A). *Sox2* is expressed throughout the entire neuroepithelium of the invaginating NP, and defines the OE at this early stage. Dual in situ hybridization experiments demonstrate that many of the *Fgf8*-expressing cells at the rim of the invaginating NP also express *Sox2* (Fig. 3.2B), as well as the definitive early OE markers *Pax6* and *Dlx5* [25]. This observation, combined with the fact that apoptosis of this cell population subsequent to loss of *Fgf8* leads to termination of OE neurogenesis and nasal cavity morphogenesis, has led to the view that these early *Sox2/Fgf8*-expressing cells are the primordial neural stem cells of the OE (Fig. 3.2C) [25].

3.3 Molecular Regulation of OE Neurogenesis In Vitro

3.3.1 FGFs Promote OE Progenitor Cell Divisions

An ongoing question for neuronal regeneration therapies has been whether most neurogenic niches fail to persist due to neurogenesis-repressive factors, or due to loss of neurogenesis-stimulating factors. Experimental manipulations that selectively ablate ORNs in adult rodents have provided evidence of increased mitotic activity by neuronal progenitors after ORN degeneration; these progenitors re-populate the OE with new ORNs. Interestingly, progenitor cell mitotic activity is re-regulated to low rates once the epithelium recovers, suggesting that progenitors are under tight negative regulation coming from the ORNs themselves [13, 15, 26–28]. Initial attempts using tissue culture methods to identify the molecular signals that regulate these events led to the discovery that the persistent neurogenesis observed in intact OE in vivo is extinguished when the tissue is moved to the culture dish. Specifically, OE progenitor cells were found to undergo terminal division in culture, producing almost exclusively ORNs, and no new progenitor cells [8]. This switch from persistent neurogenesis in vivo to terminal neurogenesis in vitro suggested the loss of a supporting signal or factor present in the surrounding environment of the epithelium. Furthermore, the similarity of this phenomenon to the observation that most other neurogenic niches switch from persistent to terminal neurogenesis around the time of birth, suggested that understanding the signals that maintain the OE's capacity for neurogenesis might shed light on why neurogenesis

Fig. 3.2 Expression of *Fgf8* and neuronal cell markers in developing OE. (A) Six successive images show in situ hybridizations for *Fgf8* (full-length ORF probe) and OE neuronal lineage markers in invaginating nasal pit (NP) at E10.5. In whole-mount in situ hybridization (left-most image), *Fgf8* is detected in commissural plate and olfactory placode, branchial arches, mid-hind-brain junction, and limb and tail buds (Scale bar, 1 mm). In serial sections, locations of neuronal lineage markers within the OE are shown: arrowheads indicate *Mash1*-expressing cells, arrow indicates *Ncam1*-expressing neurons (Scale bar, 200 μ m). (B) Double label in situ hybridization for *Fgf8* (full-length ORF probe, orange) and *Sox2* (blue) demonstrates overlap of the two markers in a small rim of surface ectoderm and adjacent invaginating neuroepithelium (brackets) (Scale bar, 50 μ m). (C) Model of peripheral-to-central process of neuronal differentiation in developing OE and origin of *Sox2*-expressing neural stem cells from *Fgf8*-expressing ectoderm. LNP, lateral nasal pit; MNP, medial nasal pit (Adapted from [25]; reprinted courtesy of Development) (See Color Plates)

in most of the mammalian nervous system does not persist throughout life. Several molecular signals responsible for promoting neurogenesis in OE have now been identified through tissue culture studies.

FGFs were the first neurogenesis-promoting factors to be identified in OE cultures. When explants of OE purified from late-gestation mouse fetuses were cultured in the presence or absence of different candidate growth factors that were known or suspected to influence proliferation of glial and/or neuronal precursors, all members of the FGF family that were tested (FGF1, -2, -4, -7, and -8) resulted in increased S-phase (³H-thymidine incorporation) labeling indices compared to untreated controls.

Detailed analysis demonstrated that FGFs act to increase the number of divisions through which INPs can progress, prior to terminal differentiation. All INPs – regardless of the number of divisions they have completed – ultimately undergo terminal differentiation to become NCAM-expressing ORNs. This indicates that INPs act as transit amplifying progenitors in the ORN lineage [1, 29, 30].

3.3.2 Stromal Cells Are Required for Proliferation and Neuronal Differentiation of OE Stem Cells In Vitro

The support of OE neurogenesis in vitro through addition of exogenous FGFs suggests that FGFs, or factors with similar action, may be produced by cells or tissues surrounding the OE to support OE neurogenesis in vivo. Identification of such factors could be vital for clinical efforts aimed at stimulating neuronal regeneration from endogenous populations of stem/progenitor cells. The experiments detailed below have shown that olfactory stromal cells secrete factor(s) capable of supporting OE neurogenesis in vitro.

An interesting finding from the series of experiments that tested actions of FGFs on OE neurogenesis in vitro was the observation that there is a rare population of progenitors within the OE that undergoes continual cell division in vitro, but only in the presence of FGFs [13, 29]. Importantly, these cells are capable of producing ORNs, confirming that they are neuronal progenitors. However, both the rarity of these cells, and their capacity for prolonged division in the presence of FGFs, suggested that they represent an early stem or progenitor cell of the OE, possibly the cell population that underlies the persistent neurogenesis observed within the OE in vivo.

To facilitate examination of potential OE neural stem cells, a more direct approach was developed to isolate and culture them [31]. This procedure, called the neuronal colony-forming assay, is illustrated in Fig. 3.3. Immunological panning of a dissociated neuronal cell fraction of OE explants was performed using anti-NCAM-treated Petri dishes (in order to remove postmitotic ORNs), generating a relatively pure (>96%) population of NCAM⁻ ORN progenitors [27, 31, 32]. Survival of purified neuronal progenitors was dependent on them being cultured over monolayers of feeder cells harvested from the stroma that normally underlies the OE in vivo. Over approximately 1 week in culture, a small fraction of these purified progenitor cells (approximately 1 in 1,000) continued to produce small colonies of cells that contain both proliferating progenitors and differentiated, NCAM-expressing ORNs [31]. Thus, neural stem cells of the OE can be cultured for relatively long periods in vitro, and can produce differentiated ORNs, but their survival and production of downstream progenitors is dependent upon factors present in the OE microenvironment in vivo (i.e. stromal cells) [33–35]. Moreover, using this colony-forming assay, it has been possible to quantify effects of many different signaling molecules on OE neurogenesis in vitro, and then to move on to test the roles for these molecules in vivo.

Fig. 3.3 Anti-NCAM immunological panning results in pure population of OE neuronal progenitor cells. OE neuronal cell fractions were resuspended in culture medium and incubated on panning plates for 30 minutes at room temperature in the dark, with intermittent agitation. Panning plates were prepared by coating 100-mm Petri dishes with purified culture supernatant from H28 rat anti-NCAM hybridoma cells. After 30 minutes of immunological panning, cells remaining in suspension were collected, centrifuged, resuspended in culture medium, and plated at various densities on stromal cell feeder layers. The resulting population consists of >96% pure neuronal progenitor cells (*See Color Plates*)

3.3.3 *Excess ORNs Inhibit Neurogenesis by Purified OE Stem/Progenitor Cells*

In healthy OE, the epithelium undergoes low rates of proliferation and differentiation to replace loss of ORNs due to normal environmental insult (virus, noxious fumes, etc.). However, when the OE sustains massive levels of ORN death by chemical, pharmacological, or surgical (olfactory bulbectomy) manipulation, or naturally through exposure to toxins, progenitor cells respond with a large burst of proliferation [11, 13, 15]. The proliferating progenitor cells generate new ORNs until the OE returns to approximately 70% of its original thickness, after which proliferation rates return to nearly pre-lesioning levels [13, 15, 27]. Over time, the recovering OE regains its correct odorant receptor expression patterns [28]. These experiments demonstrate multiple distinct features of OE neurogenesis *in vivo*. First, neurogenesis is maintained at a low level, or even repressed, during normal “healthy” conditions. Second, OE neurogenesis is stimulated, or de-repressed, immediately following ORN cell death. Third, as the OE is repopulated with new ORNs, stimulation of neurogenesis ceases, or repression is re-instated. These features of experimentally-induced neurogenesis suggest that when ORN death occurs, (1) a neurogenesis-stimulating factor is expressed or released, or (2) a neurogenesis-repressing factor is lost or decreased. Similarly, when ORNs are regenerated, (1) the neurogenesis-stimulating factor is lost, or (2) the neurogenesis-repressing factor is reinstated.

To determine whether ORNs secrete a factor that has downstream effects on neurogenesis, purified OE stem/progenitor cells were cultured in the presence of excess ORNs. This resulted in a significant *decrease* in the level of neurogenesis [31], clearly indicating that ORNs provide negative feedback to OE progenitor cells through some neurogenesis-repressing factor(s). Interestingly, neurogenesis in these assays is not inhibited by a similar excess of OE stromal cells, suggesting that the repressive factor is specifically produced by differentiated ORNs [31].

3.3.4 *Negative Regulation of OE Neurogenesis In Vitro by BMPs*

The inhibitory effect of differentiated ORNs on neurogenesis suggests the secretion, by ORNs, of a neurogenesis-inhibiting factor that acts upon the neural stem/progenitor cells that underlie ORNs in the OE *in vivo* (Fig. 3.1B). Identification of such inhibitory factors could contribute to development of regenerative therapeutic efforts, since removing or antagonizing such factors could significantly increase the efficacy of regenerative therapies.

Bone morphogenetic proteins (BMPs), the largest group of ligands in the TGF- β superfamily, were initially investigated as candidates for molecules that act as negative regulators of OE neurogenesis, for several reasons: BMPs and their receptors

are expressed in appropriate regions of the embryonic OE and/or olfactory placode to play a role in regulating OE neurogenesis [36–39]. Furthermore, studies indicate that in other neurogenic niches, BMPs seem to function as neurogenesis-inhibiting signals from the earliest stages of neural development [40–44].

In initial investigations of BMPs, neuronal colony-forming assays were performed in which purified OE neuronal stem/progenitor cells were cultured on stromal feeder layers in the presence or absence of BMPs. BMP4 addition at the time of progenitor cell plating resulted in inhibition of neuronal colony formation [45]. Interestingly, both BMP2, a close relative of BMP4 [46]; and BMP7, a more distantly-related BMP; had equally inhibitory effects [45]. These findings suggest an important role for all three BMP family members in neurogenesis.

To determine whether BMPs act specifically on proliferation of certain progenitor cell types, OE explant cultures were used: progenitor cells proliferate and can be identified easily in such cultures [8, 13, 29]. These studies demonstrated that addition of BMP4 to cultures caused a dramatic reduction in ^3H -TdR incorporation by neuronal progenitors, compared to control (untreated) cultures (Fig. 3.4A) [45]. Further experiments showed that this reduction in cell proliferation was due to a failure in development of MASH1⁺ neuronal progenitors (Fig. 3.4B). This reduction in MASH1⁺ cells in the explant cultures was not due to cell death (as no increase in apoptosis could be found), but by the stimulation of rapid, proteasome-mediated degradation of existing MASH1 protein [45]. This in turn resulted in a cessation of division by MASH1-expressing progenitors, and subsequent failure of the entire neuronal lineage downstream of the MASH1-expressing cell stage [45].

Interestingly, recent work by others has substantiated and extended these findings. The multiple zinc finger transcription factor *Zfp423/OAZ* (*O/E* associated zinc finger

Fig. 3.4 Effects of BMP4 on OE neuronal progenitor cells and ORNs. (A) OE explants were cultured for a total of 20 hours in the presence or absence of BMP4 (10 ng/ml), with 1.5 $\mu\text{Ci/ml}$ ^3H -TdR added for the final 6 hours. The percentage of cells that were ^3H -TdR-positive (^3H]TdR⁺) was determined as the fraction of total migratory cells surrounding each explant that had > five silver grains over the nucleus. Approximately 5,000 migratory cells were counted in each condition. Data are plotted as mean $\pm$ s.e. (B) Fluorescence and phase-contrast photomicrographs of explant cultures grown for a total of 8 hours in vitro, with or without BMP4 (20 ng/ml) added for the final 2 hours. In control conditions (Ctrl), arrow indicates a cluster of migratory neuronal progenitor cells expressing MASH1; arrowheads indicate examples of individual MASH1-positive cells. In BMP4 (BMP4), no cells have detectable MASH1 immunofluorescence (Scale, 20 μm) (Adapted from [35, 45]; reprinted courtesy of Nature Neuroscience and Development)

protein) has been shown to be a key regulator of ORN maturation that functions downstream of *Mash1* [47]. Examination of *OAZ*^{-/-} mice showed that OAZ disruption does not affect OE progenitor proliferation, but leads to decreased mature ORNs, impaired axonal targeting and increased apoptosis. Importantly, reintroduction of *OAZ* expression within the mature neuronal layer was sufficient to induce an immature ORN phenotype [47]. OAZ has also been identified as a cofactor of Smad proteins in BMP-signaling [48–50], suggesting that OAZ may play a critical role in integrating extracellular BMP signaling and intracellular transcription factor expression at various stages of ORN differentiation.

3.3.5 Low Concentrations of BMPs Can Promote the Generation of ORNs In Vitro

As discussed above, both administration of BMPs, and addition of excess ORNs, lead to decreased OE neurogenesis in culture. The detection of *Bmp4* and *Bmp7* mRNAs within the OE supports the possibility that BMPs might contribute to the neurogenesis-repressing effect of excess ORNs in culture [35]. Such an effect could have important implications for regenerative therapeutic efforts. If ORNs repress neurogenesis in culture by secreting BMPs, then antagonizing BMP activity by adding secreted protein antagonists, such as noggin [51], to the culture medium should remove the repression of neurogenesis and restore the number of neuronal colonies formed. Surprisingly, addition of noggin alone had a strong inhibitory effect on neurogenesis in neuronal colony-forming assays, even in the absence of any excess ORNs or added BMPs (Fig. 3.5) [35]. Because noggin does not itself signal, but rather binds to and inhibits BMPs from signaling [51, 52], these results suggested that one or more endogenous BMPs (already produced within the neuronal colony-forming assay cultures) promotes neurogenesis. The detection of *Bmp2*, *Bmp4*, and *Bmp7* mRNAs within OE stromal cells supports the possibility that BMPs secreted by stromal cells may support neurogenesis within the OE [35]. In fact, previous experiments had determined that the survival of purified neuronal progenitors depends on factors released from the stromal feeder layer on which they are cultured, since absence of this feeder layer results in stem and progenitor cell death [31].

To determine whether BMPs are responsible for the support of neuronal colony formation provided by stromal cell co-cultures, colony-forming assay cultures were supplemented with conditioned medium from pure stromal cell cultures that had either been (1) pre-cleared of any BMPs using beads coated with recombinant noggin (if any BMPs are responsible for the stimulatory effect of stromal cell conditioned medium, then pre-clearing BMPs from the medium with conjugated noggin should abolish that effect); or (2) control “mock-depleted” (i.e. the medium would still contain any BMPs present, and should still stimulate neurogenesis) conditioned medium. As expected, mock-depleted conditioned medium produced a significant

Fig. 3.5 Inhibition of neuronal colony formation by noggin. Numbers of neuronal colonies were normalized to the control (no noggin added) value in a given experiment. These values were expressed as the mean for two independent experiments in a given condition ($\pm$ range). Asterisk indicates that no neuronal colonies were observed when cultures were treated with 30 ng/ml noggin (Adapted from [35]; reprinted courtesy of Development)

increase in neuronal colony formation when added to colony-forming assays. When noggin-depleted conditioned medium was tested on colony-forming assays, it produced a significantly smaller increase in neuronal colony formation. These findings demonstrated that a portion of the neurogenesis-stimulating signal from stromal cells must derive from BMPs.

3.3.6 Concentration-Dependent Effects of BMPs on Neurogenesis

BMPs have been found previously to produce stimulatory effects on neurogenesis in systems other than OE [40, 43, 53–59]. Interestingly, a detailed study of BMP effects on OE cultures found certain BMPs to be capable of stimulating neurogenesis in OE cultures, when given at very low concentrations. For example, BMP4 produced a significant increase in neuronal colony formation when added to cultures at a concentration of 0.1 ng/ml, indicating that BMP4 can produce opposing, concentration-dependent effects upon OE neurogenesis (promoting at low concentrations; inhibiting at higher concentrations) [35].

How do low levels of BMP4 act to stimulate neurogenesis in neuronal colony-forming assays? The simplest explanation would be that BMP4 stimulates proliferation of OE neuronal progenitors, but this has not been substantiated despite numerous attempts and approaches. Interestingly, it was noted that treatment with 0.1 ng/ml BMP4 improved the appearance of explants, producing more healthy-looking ORNs (Fig. 3.6). Further experiments, using pulse-chase ^3H -TdR incorporation paradigms, demonstrated that whereas untreated explant cultures contain very few newly-differentiated NCAM $^+$ ORNs after 4 days in vitro (DIV) [27], cultures treated with low concentrations of BMP4 maintain their ORN population (Fig. 3.6), indicating that BMP4 *promotes the survival* of ORNs at low (Fig. 3.6) but not high (Fig. 3.4) concentrations [35].

Fig. 3.6 Low-dose BMP4 has a direct effect on olfactory neurogenesis. OE explants were cultured for a total of 96 hours in the presence or absence of 0.1 ng/ml BMP4, and cultures fixed and processed for NCAM immunoreactivity. Fluorescence photomicrographs of OE explants in the two conditions, showing increased numbers of NCAM-positive ORNs surrounding the explant in the presence of BMP4 (Scale bar, 50 μm). Low-dose BMP4 also promotes survival of newly-generated ORNs, quantified from OE explant cultures treated with both BMP4 (0.1 ng/ml) and ^3H -TdR. A significant difference in the number of surviving ORNs was observed between control and BMP4-treated cultures after 4 days in vitro ($P = 0.02$, Student's t -test) (From [45]; reprinted courtesy of Nature Neuroscience) (See Color Plates)

3.4 Negative Regulation of Neurogenesis In Vivo and In Vitro

Over 40 years ago, Bullough put forward the hypothesis that tissues produce growth-inhibitory signals, the local concentrations of which directly reflect the mass of the tissue in which they are produced [60]. Such signals were hypothesized to halt cell proliferation when appropriate tissue size had been reached, thereby maintaining cell number appropriate for a tissue's function. Identification of growth and differentiation factor 8 (GDF8)/myostatin, a signaling molecule of the activin/TGF- β group of the TGF- β superfamily, as an endogenous negative regulator of skeletal muscle development [61, 62]; and growth and differentiation factor 11 (GDF11), a TGF- β closely related in structure to GDF8, as an endogenous negative regulator of neurogenesis in OE [63]; have validated this idea [64]. Indeed, we have found that such feedback inhibition of neurogenesis is important for maintaining neuron number in two different sensory neuroepithelia in mice: the OE and the neural retina [63, 65].

3.4.1 Negative Autoregulation of OE Neurogenesis

Other endogenous factors also contribute to the regulation of OE neurogenesis. Members of both the FGF and TGF- β superfamilies have recently been shown to be expressed in relevant areas of the OE to regulate neurogenesis during development. GDF11, a recently-identified member of the TGF- β superfamily, is expressed in mouse OE beginning at E10.5, and its expression continues through development and into adulthood [18, 63]. Because the closely-related GDF8/myostatin had been shown previously to inhibit the proliferation of muscle progenitor cells [61, 66–68], it was hypothesized that GDF11 might serve a similar role within the OE. Detailed analysis of GDF11 expression showed that *Gdf11* mRNA within the nasal mucosa was confined to the OE proper, with no expression in adjacent respiratory epithelium or underlying stroma. Within the developing OE, *Gdf11* expression is confined to the basal portion of the OE where immature ORNs and their progenitors are localized (Fig. 3.1). Interestingly, the soluble GDF11 signaling antagonist *Follistatin* (*Fst*) was also found to be expressed throughout the developing OE, as well as the underlying stroma [63]. Examination of *Gdf11* mRNA within the OE of *Mash1*^{-/-} mice (in which INPs and ORNs are absent) found that *Gdf11* expression is essentially lost in the absence of *Mash1* (Fig. 3.7) [63]. Because the OE of *Mash1*^{-/-} mice becomes populated by sustentacular cells and stem cells, but is devoid of ORNs and INPs, *Gdf11* must be expressed by either ORNs themselves or the INPs that are also lost in *Mash1*^{-/-} mice [69].

Does GDF11 affect OE progenitor cell proliferation or survival? Using similar in vitro assays to those described previously, GDF11 was found to inhibit proliferation of OE neuronal progenitor cells, as assayed by ³H-TdR incorporation. Interestingly, GDF11 had no effect on MASH1 expression, nor did it inhibit the

Fig. 3.7 *Gdf11* is expressed by ORNs and their progenitors. OE from E17.5 *Mash1*^{-/-} embryos and wild-type littermate was hybridized with probes to *Ncam*, *Gdf11*, *Ngn1*, and *Mash1*. In *Mash1*^{-/-} mice the ORN lineage is cut short at an early stage, as *Mash1*-expressing neuronal progenitors initially form, but then undergo apoptosis. Thus, the OE of *Mash1*^{-/-} mice is markedly thinner than that of wild-types, and expression of *Ngn1* and *Ncam* is drastically reduced since the epithelium lacks most ORNs and ORN progenitors. *Gdf11* expression is also essentially absent. Since sustentacular cells and horizontal basal cells are still present in *Mash1*^{-/-} mice, this indicates that the cells that normally express *Gdf11* must be ORNs and ORN progenitors (Scale, 20 μ m; AP, apical surface; BL, basal lamina; LP, lamina propria) (From [63]; reprinted courtesy of Neuron) (See Color Plates)

proliferation of *Mash1*-expressing progenitor cells [63], as was found previously for BMP4 [45]. If GDF11 decreases progenitor proliferation in OE explant cultures, but is not acting on MASH1-expressing progenitor cells, then its next most likely site of action is upon the progeny of MASH1-expressing cells – the INPs. Previous studies [29, 35] found that INP proliferation is stimulated by FGF2 treatment. Addition of both FGF2 and GDF11 completely abolished INP proliferation, suggesting that the stimulatory effect of FGF2 on INP proliferation is abrogated by GDF11's effects. GDF11 does not affect INP cell survival, but the increase in expression of the cyclin-dependent kinase inhibitor p27^{Kip1} in these cultures suggests that GDF11 halts INP progression through the cell cycle [63].

Is GDF11 the endogenous factor responsible for feedback inhibition of OE neurogenesis *in vivo*? To answer this question, we performed genetic experiments in which mice, homozygous for a null allele of *Gdf11*, were analyzed for OE neurogenesis [63]. These genetic experiments confirmed the *in vitro* results: examination of BrdU incorporation in *Gdf11*-null mice showed significantly higher numbers of BrdU⁺ cells within the developing OE compared to wild-type animals, reflecting increased OE neurogenesis in the absence of the antineurogenic activity of GDF11. Furthermore, significantly *decreased* numbers of BrdU⁺ cells were observed within the OE of *Fst*-null mice compared to wild-types, reflecting the increase in GDF11 activity resulting from absence of its endogenous antagonist [63]. Interestingly, *Ngn1* expression within the OE was significantly increased in *Gdf11*-null mice, reflecting increased production of INPs in the absence of GDF11 activity. *Mash1*

expression, which marks the progenitor cells that precede INPs within the OE lineage, were unaffected in *Gdf11*-null mice, further supporting the idea that GDF11 acts specifically upon INPs, and not on earlier cell types within the OE lineage [63].

3.4.2 Regulation of Progenitor Cell Competence by GDF11 in Neural Retina

The OE differs from most other mammalian neurogenic tissues in its ability to continually regenerate throughout life. The discovery that GDF11 signaling provides negative autoregulation of OE neurogenesis to allow regeneration, but prevent overgrowth, suggests that a similar signaling mechanism may actively repress neurogenesis within neural tissues considered to be non-regenerative. If this is the case, repressing GDF11 action may unlock a tissue's regenerative potential. Interestingly, both GDF11 and FST are expressed in various areas of the CNS [65, 70–72], such as the dentate gyrus of the hippocampus, the external granule layer of the cerebellum, and the retina. Specifically, recent investigation of the development of the neural retina (considered to be a non-regenerative sensory epithelium) has provided evidence that GDF11/FST signaling plays a unique and important role in the regulation of neural retinal neurogenesis during development [65].

Whereas the ORNs of the OE develop through sequential progression of the OE neural stem cell through various progenitor states, the mammalian retina consists of seven distinct neural cell types, which are all derived from one population of multipotent retinal progenitors [73, 74]. These cells are generated at various stages of retinal development, as the progenitor cells pass through a stereotyped pattern of “competence states” [75–77]. Nearly all retinal cell types are generated prenatally, and retinal neurogenesis in mice ceases completely during the first 2 weeks of life. The production of the various cell types of the retina is controlled by specific expression patterns of various homeobox and basic helix-loop-helix (bHLH) transcription factors [reviewed in 78]. However, the mechanisms governing the expression of these proneural genes are poorly understood.

The identification of GDF11 as a negative regulator of OE neurogenesis [63], and its expression within the developing retina [71], suggested that it might play a role in the regulation of retinal neurogenesis. The *Gdf11* transcript is first expressed in the retina around E12.5 in mice (Fig. 3.8A), when retinal ganglion cells (RGCs) first begin to differentiate [65]. It is expressed throughout the retina, with highest expression in the ganglion cell layer at E15.5, and continues after birth. *Fst* is first detected at E13.5, and similarly exhibits its highest expression within the ganglion cell layer from E15.5 on (Fig. 3.8A). Interestingly, mutant retinas from *Gdf11*-null mice showed obvious morphologic abnormalities, characterized by abnormally high cell density within the ganglion cell layer, and complete lack of the inner plexiform layer (Fig. 3.8B) [65]. Examination of cell marker expression showed expanded expression of the RGC marker *Brn3b*, suggesting that development of this particular cell type is affected in the absence of *Gdf11* (Fig. 3.8B). Furthermore,

decreased expression of *Crx1* and *Prox1* transcripts indicated deficient production of early photoreceptors and amacrine cells, respectively [65].

The changes observed within the retinas of *Gdf11*-null animals are reminiscent of those observed within the OE, suggesting that GDF11 is a negative regulator of RGC neurogenesis. However, whereas the *Gdf11*-null OE exhibited significant changes in overall thickness, and in progenitor cell proliferation [63], the retina exhibited no change in overall thickness (Fig. 3.8C) or retinal progenitor proliferation [65]. Because proliferation appears unaffected, the temporal period of RGC genesis was examined via BrdU birthdating, and was found to be significantly expanded (in terms of BrdU-retaining cell number within RGC layer) in the *Gdf11*-null retina (Fig. 3.8C). Experiments using in vitro cultures have shown that GDF11 decreases production of RGCs and increases production of later born cell types: retinal explants cultured in the presence of exogenous GDF11 showed decreased expression of *Brn3b*, and increased expression of *Crx1*. Interestingly, although the RGC population is abnormally expanded in *Gdf11*-null retinas, RGCs appear to differentiate normally, extending axons through the optic chiasm and tracts, which are also abnormally thick (Fig. 3.8D). Neurofilament immunohistochemistry demonstrated an estimated 37% increase in cross-sectional areas of optic nerves in *Gdf11*-null animals compared to wild-types (Fig. 3.8E) [65]. Importantly, the ability of these cells to develop and extend axons along the correct path suggests that they likely can form appropriate connections, often a significant barrier to successful regeneration.

Because retinal progenitor cell proliferation is unchanged in *Gdf11*-null retinas, it appeared that GDF11 influences the competence state of retinal progenitors to produce specific cell types. It was hypothesized that if GDF11 can directly control progenitor cell competence, it might exert such changes through altered expression patterns of the transcription factors that determine competence state. One of the bHLH factors essential to RGC development is *Math5*: in the absence of *Math5* RGC production is severely reduced, and amacrine cell production is increased [79–81]. In *Gdf11*-null retinas, *Math5* expression begins normally, but remains high for an abnormally long period of development, corresponding to the period of prolonged RGC production [65]. The altered expression of *Math5* is accompanied by a delay in the onset of *Mash1* and *NeuroD1*, two transcription factors important for the development of bipolar and amacrine cells [82, 83]. Conversely, *Math5* is prematurely down-regulated in *Fst*-null retinas, and *Mash1* expression was detected earlier [65].

3.5 Conclusions and Future Directions

The findings discussed above indicate that, during embryonic development, GDF11 expression within the retina regulates the timing of progenitor cell competence by controlling the expression of genes involved in progenitor cell fate determination (Fig. 3.9). Whereas GDF11 signaling within the OE regulates the production of ORNs through its actions on one specific cell within the OE progenitor cell lineage, its actions within the retina are more complex, affecting a number of cell types,

both positively and negatively, by influencing transcription factor expression. In so doing, GDF11 signaling regulates the numbers of specific retinal cell types required to produce a functioning retina. Could GDF11 signaling play a role in the early postnatal switch from continued proliferation of retinal progenitor cells to terminal differentiation? The perinatal lethality of *Gdf11*-null mice has not allowed us to answer this question. However, preliminary attempts at retina-specific disruption of *Fst* indicate that GDF11 signaling continues to play an important role in the regulation of progenitor cells in the postnatal retina [84]. Furthermore, the discovery of a population of retinal stem cells that remains dormant in the ciliary marginal zone well into adulthood [85] suggests the potential to induce endogenous retinal stem cells to divide and regenerate in damaged tissue. Finally, attempts to guide cultured stem or progenitor cells toward particular phenotypes for use in transplantation therapy [86, 87] will be greatly aided by better understanding the molecular signaling that controls the generation and specification of particular cell types. Future experiments aimed at modulation of GDF11 signaling within the retina, both through genetic and pharmacologic means, should provide significant insight into whether retinal stem and progenitor cells can be utilized within the postnatal eye to promote measurable recovery from debilitating injuries and diseases of the eye.

Interestingly, GDF11 has been reported to play a role in early development of a number of tissues, including pancreas [88], kidney [89, 90], muscle [91], bone [91], and spinal cord [92]. Within spinal cord, GDF11 contributes to neuronal subtype specification, similar to its role in retina [92]. GDF11 expression has also been shown to persist into adulthood in discrete regions of the CNS, such as the dentate gyrus of the hippocampus and the external granular layer of the cerebellum [63, 65]. Identifying the role of GDF11 signaling within these regions both during development and in adulthood could have critical implications for the study of neuronal regeneration within the cerebellum and hippocampus.

Current efforts at clinical therapies using stem or progenitor cells to treat neurodegenerative diseases fall under two major themes: (1) directing cultured stem cells to a neural fate and subsequently implanting the derived neural progenitors into areas of need; or (2) focal revival of endogenous stem cells to repopulate damaged areas. Presently, these efforts are significantly hindered by a limited understanding of the

Fig. 3.8 *Gdf11* mutants exhibit retinal abnormalities. (A) ISH for *Gdf11* and *Fst* in developing mouse retina; nbl, neuroblastic layer; gcl, ganglion cell layer. Arrow in inset indicates *Fst* expression in presumptive amacrine cells (Scale bars, 200 μ m) (B) Left, hematoxylin-eosin-stained paraffin sections of retina. Right, ISH for *Brn3b*. Insets, higher magnification of *Brn3b*⁺ gcl (Scale bars, 100 μ m) (C) Top, increased cell number ($P < 0.01$, student's *t*-test) in *Gdf11*-null retinas. Total cell nuclei in GCL + IPL were counted in 300 μ m of central retina in P0 cryosections stained with Hoechst. Bottom, no significant change in central retina thickness. Histograms show mean $\pm$ SEM of measurements from four to five animals of each genotype. (D) β -galactosidase (X-gal) staining of sections of *Gdf11*-null- and *Gdf11*^{+/+}-*Tattler-1* embryos (Scale bars, 200 μ m; on, optic nerve; oc, optic chiasm) (E) Cross sections of dissected optic nerves stained with antibodies to neurofilament (Scale bar, 50 μ m) (From [65]; reprinted courtesy of Science) (See Color Plates)

Fig. 3.9 Contrasting roles of GDF11 signaling in OE and retina neurogenesis. In developing OE, GDF11 negatively regulates neuronal production by reversibly blocking the division of *Ngn1*-expressing INPs through increased expression of the cyclin-dependent kinase inhibitor p27^{Kip1}. Loss of ORNs releases INPs from the GDF11-mediated negative regulation, allowing them to increase their proliferation until neuron number is restored. In contrast, GDF11 signaling in retinal neurogenesis specifies retinal cell type specification by regulating retinal progenitor cell competence state. GDF11 suppresses *Math5* expression, and promotes expression of various homeodomain genes such as *Pax6* and bHLH factors such as *NeuroD*, driving progenitors to acquire competence to produce later-born cell types such as amacrine cells and photoreceptors (Adapted from [63, 78] ; reprinted courtesy of Neuron and Genes and Development) (See Color Plates)

molecules that regulate stem cell activity, both in terms of endogenous control of stem cells in vivo, and manipulation of cultured stem cells in vitro. Gaining a better understanding of their origin, proliferation, maturation, and phenotypic specification will improve the efficacy of such therapies by increasing the efficiency of generating specific cell types or tissues. Studies of the regulation of olfactory and retinal neurogenesis provide a molecular foundation for future attempts at guiding stem and progenitor cells toward specific cell fates for use in clinical therapies, with the hope that we may eventually harness the full potential of endogenous stem and progenitor cells that might otherwise be incapable of further growth and regeneration.

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