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# Chapter 10 Feedback Regulation of Neurogenesis in the Mammalian Olfactory Epithelium: New Insights from Genetics and Systems Biology

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## 10.1. INTRODUCTION

### 10.1.1. Ongoing Neurogenesis in the Olfactory Epithelium (OE) in Vivo and in Vitro

The mouse olfactory epithelium (OE) is an ideal model system for identifying and characterizing the factors that regulate proliferation and differentiation of neurons from their stem and progenitor cells. In part, this is because the OE undergoes neurogenesis throughout life, and does so exuberantly in response to injury (Graziadei and Monti Graziadei 1978; Mackay-Sim and Kittel 1991; Calof et al. 2002). However, another advantage of great significance is the fact that numerous studies have given us a good idea of the cell types that give rise to olfactory receptor neurons (ORNs) (Cau et al. 1997; Calof et al. 2002; Kawauchi et al. 2004, 2005; Beites et al. 2005; see also Chapter 5). Thus, in the neuronal lineage of the OE, four cell stages have been identified, in vitro and in vivo: (1) *Sox2*-expressing stem cells, which reside in the basal compartment of the epithelium, are thought to commit to the ORN lineage via expression of the proneural gene, *Mash1*. (2) *Mash1*-expressing early progenitor cells, which divide and may act as transit-amplifying cells (Gordon et al. 1995), in turn give rise to (3) late-stage transit-amplifying cells, also known as immediate neuronal precursors (INPs), which express a second proneural gene, *Ngn1* (Wu et al. 2003). INPs give rise to daughter cells that undergo terminal differentiation into (4) postmitotic *Ncam*-expressing ORNs. Figure 10.1A shows schematics of both the OE neuronal lineage and the spatial distribution of these cells within the OE in vivo. As is common to many epithelia, differentiation in the OE proceeds in a basal-to-apical direction: dividing stem and progenitor cells lie atop the basal lamina, and multiple layers of differentiated ORNs lie above the progenitor cells layers.

Since the OE is able to sustain de novo neurogenesis throughout life and to regenerate in response to injury (Graziadei and Monti Graziadei 1978; Calof et al. 2002), it must contain stem cells. Indeed, several groups have been interested in harvesting OE stem cells for their therapeutic potential (e.g., Zhang et al. 2004; Othman et al. 2005). However, when OE is isolated and cultured in serum-free medium, although it avidly generates neurons for one to two days (Calof and Chikaraishi 1989), it rapidly loses the ability to undergo neurogenesis unless other factors or feeder cells are added (DeHamer et al. 1994; Holcomb et al. 1995; Mumm et al. 1996; Shou et al. 2000). In other words, OE neuronal stem and transit-amplifying cells in isolation are prone to undergoing differentiative divisions over self-replicative divisions, resulting in rapid expiration of these cell populations in tissue culture. This observation has prompted numerous studies to search for the environmental cues that are important for sustaining stem and progenitor cell self-renewal and maintaining the neurogenic potential of the OE.

### 10.1.2. Regeneration in the Olfactory Epithelium (OE) Follows a Specific Time Course

Ultimately, regeneration in the OE is a mechanism for producing neurons when neurons are lost. Several injury models have been used to study neuronal regeneration in the OE. One of these, methyl bromide inhalation, in which exposure to methyl bromide gas damages all cell types in the OE and adjacent respiratory epithelium nonselectively, is not considered in this chapter (for details, see Schwob et al. 1995, 2002; Huard et al. 1998; Jang et al. 2003). Probably the most selective surgical procedure for inducing neuronal degeneration and subsequent neurogenesis in the OE of rodents is surgical removal of one of the two olfactory bulbs (OBs) of the brain (unilateral "bulbectomy," schematized in Figure 10.1B; since the OBs are the direct synaptic targets of ORNs, bulbectomy severs ORN axons). Unilateral bulbectomy causes a selective degeneration of ORNs, and numerous studies have shown that it results in a synchronous wave of apoptosis in the ORN population in the OE ipsilateral to the lesion, followed by near-complete regeneration of the OE

over a stereotyped time course (Costanzo and Graziadei 1983; Schwartz Levey et al. 1991; Carr and Farbman 1992; Schwob et al. 1992; Holcomb et al. 1995; Leung et al. 2007; Iwai et al. 2008).

The details of neuronal regeneration following bulbectomy involve the proliferation and differentiation of a defined sequence of cellular intermediates, most of which appear to be the same cell types that have been identified in tissue-culture studies of OE neurogenesis and during OE development. Induction of ORN death, which peaks at about two days post-bulbectomy (Holcomb et al. 1995), leads to increased replicative and differentiative divisions of *Mash1*-expressing progenitors and their progeny, the INPs (schematized in Figure 10.1C; cf. Schwartz Levey et al. 1991; Gordon et al. 1995; Holcomb et al. 1995). As new ORNs are generated, the rate of progenitor cell divisions decreases until steady state is restored, about ten days after surgery in bulbectomized mice (Schwob et al. 1992; Holcomb et al. 1995; Calof et al. 1996a). These observations suggest that progenitor cells in the OE are able to "count" the number of ORNs present in the epithelium, and respond by altering their rates of division and differentiation appropriately. Indeed, tissue-culture studies have shown that purified OE neuronal progenitor cells, whose ability to generate ORNs can be quantified in neuronal "colony-forming assays," show reduced levels of neurogenesis when grown in the presence of large numbers of ORNS, indicating that ORNs produce a signal(s) that inhibits neurogenesis by their own progenitors (Figure 10.2A; Mumm et al. 1996).

This ability to "sense" changes in ORN number is presumably essential for the OE's ability to respond to fluctuations of neuronal number that occur as the OE is subjected to infection and toxic insults during the normal course of life (Hinds et al. 1984; Mackay-Sim and Kittel 1991). As described below, the response to changes in ORN number appears to be mediated by a network of signaling molecules that are expressed by, and act upon, cells within the OE itself. Recent studies using mouse genetics and tissue-culture approaches, as well as computational modeling, have begun to shed light on how the integrated action of these endogenous signaling molecules, as well as their interaction with transcriptional effectors such as *Foxg1,* coordinate replicative and differentiative divisions of OE stem and progenitor cells to control both the sizes of different OE neuronal cell populations and the morphogenesis of the olfactory mucosa and nasal cavity.

## 10.2. ENDOGENOUSLY EXPRESSED SIGNALING MOLECULES REGULATE ONSET AND MAINTENANCE OF NEUROGENESIS

### 10.2.1. Mesenchyme-Derived Factors Sustain Prolonged Neurogenesis in Vitro

Although the OE is able to sustain de novo neurogenesis throughout life, cultured OE rapidly loses its ability to produce neurons. In serum-free medium, cultured OE stem and progenitor cells undergo differentiative divisions rather than replicative divisions, leading to depletion of stem and progenitor cells (Calof and Chikaraishi 1989). To identify conditions that would lead to sustained stem/progenitor cell activity in culture, Mumm et al. (1996) developed methods to purify (>96%) OE stem and progenitor cells by immunological "panning," depleting dissociated OE cells of ORNs using anti-NCAM antibodies immobilized on petri dishes. When these cells were cultured on top of a feeder layer consisting of cells from the olfactory stroma (mesenchyme-derived cells that underlie that OE proper), stem and progenitor cells were able to sustain proliferation and ORN production for as long as two weeks in culture (Mumm et al. 1996; Shou et al. unpublished observations). The results from this study led to the hypothesis that at least some of the factors that promote stem cell maintenance and the OE's capacity for regeneration are produced in the underlying mesenchyme (and, during postnatal life, the lamina propria of the olfactory mucosa).

### 10.2.2. FGF8 is a Positive Autocrine Regulator of Primary Olfactory Neurogenesis in Vivo

Experiments using primary OE cultures have shown that several members of the fibroblast growth factor (FGF) signaling family promote proliferation of OE stem and progenitor cells (Figure 10.2B; DeHamer et al. 1994). FGFs comprise a large family of secreted signaling proteins that have been implicated in controlling cell replication, differentiation, and survival in almost all tissues (Ornitz 2000). In OE cultures, FGFs were found to promote sustained proliferation of both stem cells and INPs. Detailed examinations demonstrated that FGFs act on INPs by preventing cell cycle exit, thereby increasing the likelihood that INPs will undergo a second round of replicative division before

undergoing terminal differentiation into NCAM-expressing ORNs (DeHamer et al. 1994).

Which FGF is responsible for stem and progenitor cell maintenance in vivo? A number of *Fgfs* are expressed in and around the OE in vivo, during development and in postnatal life (DeHamer et al. 1994; LaMantia et al. 2000; Bachler and Neubuser 2001; Hsu et al. 2001; Kawauchi et al. 2005). Molecular analyses have revealed that *Fgf8* is highly expressed at early stages of OE development, during primary neurogenesis; and that it is expressed in *Sox2*+ primordial neural stem cells in the epithelial margins of the invaginating olfactory pit (Figure 10.3A; Bachler and Neubuser 2001; Kawauchi et al. 2005). Other *Fgfs* are expressed in the OE at later times in development. For example, *Fgf18* is expressed in the OE during the final third of embryonic development (Kawauchi et al. 2005); and *Fgf2* expression within OE proper cannot be detected until postnatal life (Hsu et al. 2001; Kawauchi et al. 2004). Thus, it seems likely that expression of different FGFs occurs during different time periods, and/or in different cell types, during development, and regeneration of the OE. Moreover, these observations imply that different FGFs play different roles in controlling the proliferation, differentiation, and/or survival of different OE cell types.

Partial data exist on which FGFs are required for OE development and neurogenesis. Genetic experiments have shown that *Fgf8* is crucial for both OE neurogenesis and nasal cavity morphogenesis during embryonic development. Importantly, the role of FGF8 in these processes is not that of a mitogen. Rather, expression of *Fgf8* is required for the survival of *Sox2*-expressing primordial neural stem cells of the OE (Figure 10.3C; Kawauchi et al. 2005). These *Sox2*+ stem cells form the foundation of the OE neuronal lineage, and in the absence of *Fgf8*, they undergo apoptosis. The result of this event, which occurs during invagination of the olfactory pit at days 10–12 of gestation, is cessation of both OE neurogenesis and morphogenesis of the nasal cavity and olfactory mucosa. Mice with deletion of *Fgf8* in anterior neural regions survive to birth, but have virtually no nasal cavity and no OE (Figure 10.3; Kawauchi et al. 2005). Thus, since *Fgf8* is expressed in the very cells (*Sox2*-expressing primordial neural stem cells) whose survival it maintains, we think of FGF8 as a positive autocrine regulator of neurogenesis that acts during the initial establishment of the OE neuronal lineage.

## 10.3. REGULATION OF NEUROGENESIS BY NEGATIVE FEEDBACK

### 10.3.1. Neuronal Cell-Derived Factors Inhibit Progenitor Cell Proliferation in Vitro

Signals that mediate negative feedback of neurogenesis are as important in OE development and regeneration as those that promote neurogenesis. Although the temporal and spatial relationship between induced apoptosis of ORNs and proliferation of progenitor cells/ORN regeneration (Figure 10.1) suggest that those cells of the OE that are more differentiated (i.e., INPs and ORNs) feed back to inhibit proliferation and neuron genesis by proliferating progenitor cells, this concept was not tested directly until about 13 years ago. Mumm and colleagues performed experiments in which they showed that adding large numbers of neuronal cells (comprised of approximately 75% ORNs) to cultures of isolated OE neuronal progenitor cells suppresses neurogenesis by the isolated progenitors (Figure 10.2A; Mumm et al. 1996). Additional biochemical tests indicated that the neuronal cell-derived signal was a polypeptide (Calof et al. 1996b), and led to the examination of transforming growth factor (TGF-fj) superfamily signaling molecules as candidates for the antineu-rogenic feedback factor(s) of the OE.

### 10.3.2. Autoregulation of Neurogenesis by GDF11

Studies using primary OE cultures have identified several members of the TGF-β family of signaling molecules as potent negative regulators of OE neurogenesis (DeHamer et al. 1994; Shou et al. 1999, 2000; Wu et al. 2003). Indeed, of the numerous signaling molecules assessed in an early screen to test for factors affecting immediate neuronal precursor (INP) proliferation (Figure 10.2B), the only factor to have a negative effect on INP proliferation was TGF-β1 (DeHamer et al. 1994). TGF-βs comprise a large superfamily of secreted signaling molecules that have been implicated in regulating proliferation, differentiation, and cancer in virtually all tissues (Hogan 1996; Massague et al. 2000; Chang et al. 2002; Feng and Derynck 2005; Liu and Niswander 2005). A number of different TGF-βs are expressed in OE proper and its underlying mesenchymal stroma, and studies have shown that these have diverse effects on OE

neurogenesis, including control of development of *Mash1*-expressing progenitors and effects on ORN survival (Shou et al. 1999, 2000; Wu et al. 2003; Kawauchi et al. 2004).

Using a candidate approach to identify the signal(s) responsible for endogenous negative feedback of neurogenesis in the OE, Wu and colleagues focused on growth and differentiation factor 11 (GDF11), a member of the activin-like family of TGF-βs, which signal intracellularly via Smads2 and 3 (Andersson et al. 2006; Massague and Gomis 2006). A primary reason for focusing on GDF11 was its extensive homology to GDF8 (myostatin), an autocrine negative regulator of skeletal muscle cell growth (Lee and McPherron 1999; Nakashima et al. 1999; Wu et al. 2003). During development, *Gdf11*—which is expressed specifically in the OE neuroepithelium proper, and there primarily by immature ORNs and neuronal progenitors—plays a crucial role in the negative regulation of neuron number (Nakashima et al. 1999; Gamer et al. 2003; Wu et al. 2003). In vitro, GDF11 induces complete, but reversible, inhibition of INP cell divisions, without affecting development of their precursors, the *Mash1*-expressing early progenitor cells (Figure 10.4A and B; Wu et al. 2003). Interestingly, GDF11's antiproliferative effect is able to override the positive effect of FGFs on INP proliferation, which have been described previously (see above and DeHamer et al. 1994), indicating that INPs must integrate signals from the FGF and TGF-β different signaling pathways to control their proliferation and growth. INP cell cycle arrest appears to be mediated by GDF11-induced upregulation of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, which arrests cells in G1 phase (Figure 10.4D through F; Chen and Segil 1999; Levine et al. 2000; Miyazawa et al. 2000; Dyer and Cepko 2001).

Importantly, the negative regulation of neurogenesis affected by GDF11 is also observed in vivo. In mice that are made null for *Gdf11* (*Gdf11*$^{tm2/tm2}$ mice), the second reported null allele of *Gdf11* (Wu et al. 2003), the OE contains increased numbers of INPs and ORNs and shows an increase in overall thickness compared to wildtype littermates (Figure 10.4G). Just as has been shown in vitro, however, there appears to be no change in the number of *Mash1*-expressing cells in the OE of *Gdf11*-null mice (Wu et al. 2003). Thus, GDF11 acts as an autocrine negative regulator of neuron number during OE development, and its actions appear to be directed at INPs, the immediate precursors of ORNs. A schematic showing the present concept of how GDF11 acts in regulating feedback inhibition of neurogenesis is shown in Figure 10.4I.

*Gdf11* expression in the OE can be detected as early as day 10.5 of development (Kawauchi et al. 2009), and it continues to be expressed through development and adulthood. How is neurogenesis able to proceed in a tissue that expresses persistent levels of antineurogenic factors such as GDF11? Since *Gdf11* transcripts can be detected in a number of neural regions, in addition to OE, in which robust neurogenesis occurs even into postnatal periods (Nakashima et al. 1999; Kim et al. 2005; Wu and Calof unpublished observations), GDF11 activity must be tightly regulated in order for appropriate progenitor cell proliferation to be maintained and proper neuron number achieved.

### 10.3.3. Follistatin (FST), a GDF11 Antagonist, Provides a Permissive Environment for Neurogenesis

Follistatin (FST), a secreted protein, has been shown to antagonize signaling by a number of different activin-like TGF-βs, including activins themselves, GDF8, GDF11, and BMP7 (Gamer and Rosenblatt 1986; Schneyer et al. 1994, 2008; Gamer et al. 1999; Lee and McPherron 2001; Rebbapragada et al. 2003; Wu et al. 2003). *Fst,* which is expressed in OE and its underlying stroma, antagonizes activin-like TGF-βs by binding to them and preventing signaling through their receptors (Sugino et al. 1997; Phillips and de Kretser 1998; Schneyer et al. 2003). In OE cultures, the addition of FST abrogates GDF11's antiproliferative effects on INPs (Figure 10.4A), suggesting that FST's presence in vivo may be of importance for controlling the magnitude and extent of GDF11 antineurogenic signaling. Indeed, when the OE of mice null for *Fst* (*Fst*$^{-/-}$ mice; Matzuk et al. 1995) was examined, it was found to have severely decreased numbers of INPs and ORNs, as well as a much thinner OE than that observed in wildtype littermates (Figure 10.4H; Wu et al. 2003). Thus, current thinking suggests that the presence of FST within the nasal mucosa is crucial for maintaining an environment permissive for OE neurogenesis (see also Figure 10.9A; Kawauchi et al. 2009).

Recent evidence indicates that GDF11 is not the only antineurogenic factor that is regulated by FST in the OE. In

addition to INPs, both *Sox2*- and *Mash1*-expressing stem/early progenitor cells are also depleted dramatically in $Fst^{-/-}$ OE (Wu and Calof, unpublished observations). As all available data indicate that the antineurogenic effects of GDF11 are limited to cells downstream of *Mashl*-expressing progenitors in the ORN lineage, these observations suggest that another molecule, whose signaling is antagonized by FST, controls the divisions of *Sox2*- and *Mash1*-expressing early stem and progenitor cells. Accordingly, recent experiments indicate that activins themselves are expressed within the nasal mucosa, and have negative effects on stem/progenitor cell proliferation in OE cultures (Gokoffski and Calof, unpublished observations).

Although the observations described above come from studies of developing OE, *Gdf11* and *Fst* continue to be expressed in the adult (Gokoffski and Calof, unpublished observations), suggesting that they play a significant role in mediating the controlled and coordinated regeneration that is observed in injured adult OE. Testing such predictions has been limited by the fact that $Gdf11^{-/-}$ and $Fst^{-/-}$ mice die at birth, for reasons unrelated to their effects on OE (Matzuk et al. 1995; McPherron et al. 1999; Esquela and Lee 2003; Wu et al. 2003). Development of conditional mutant alleles that allow for tissue-specific inactivation of *Gdf11* and *Fst* will permit experiments to be performed that should provide important insights for understanding how regeneration is controlled and how stem/progenitor activity is coordinated with ORN number during this process (Jorgez et al. 2004). Since GDF11 has also been shown to be a regulator of stem cell fate in another sensory neuroepithelium, the retina (Kim et al. 2005), it will be interesting to see if GDF11 plays such a role in OE regeneration.

## 10.4. COMPUTATIONAL APPROACHES SUGGEST CRUCIAL ROLES FOR NEGATIVE FEEDBACK IN ACHIEVING RAPID AND ACCURATE REGENERATION IN THE OLFACTORY EPITHELIUM (OE)

### 10.4.1. GDF11 Controls the Ratio of Proliferative vs. Differentiative Divisions of Immediate Neuronal Precursor Cells

The location of the OE within the nasal cavity exposes it directly to the environment, making it vulnerable to random environmental assaults, which in turn leads to highly variable rates of ORN loss. Such unpredictability creates significant challenges for the homeostatic control of ORN number. Yet, the OE performs remarkably well: even when virtually all ORNs are eliminated acutely, ORN numbers are restored rapidly and without substantial overshoot (Schwartz Levey et al. 1991; Carr and Farbman 1992; Holcomb et al. 1995; Ducray et al. 2002; Costanzo and Graziadei 1983; Schwob et al. 1995). Since olfaction is crucial for the survival of many animals, rapid and accurate regeneration of ORNs has obvious evolutionary advantages. Can we directly relate the feedback provided by molecules produced within the OE, such as GDF11, activins, and FST, to the rapidity and accuracy of regeneration?

The question of how dynamic processes, such as feedback, enable systems to achieve goals such as robustness, efficiency, and speed, is a major focus of systems biology, and is often approached with the help of mathematical and computational modeling. We recently applied such methods to the analysis of feedback within the OE neuronal lineage (Lander et al. 2009), with striking results. The first thing we learned was that, if the sole action of GDF11 is to regulate the rate of INP cell divisions (as had been shown in vitro; Wu et al. 2003), then GDF11 could contribute nothing to steady-state homeostasis (i.e., maintaining a desired number of ORNs despite variable environmental challenges). Moreover, its contribution to increasing overall speed of regeneration would be modest at best.

Further modeling led us to predict that GDF11 has an additional action: controlling the proportion of INP daughters that become ORNs instead of continuing to divide and becoming more INPs (cf. Figure 3 in Lander et al. 2009). When tissue-culture experiments were performed to test this hypothesis directly, they demonstrated that GDF11 does indeed control INP differentiation, in a dose-dependent manner (Figure 10.5): treatment with low concentrations of GDF11 (0.1–1 ng/mL) pushes INPs to differentiate into NCAM-expressing ORNs; whereas high doses of GDF11 (20 ng/mL) in these same cultures prolongs INP cell-cycle length, delaying differentiation of these cells to ORNs (Figure 10.5). Significantly, these actions of GDF11 occurred over the same time course that was predicted from modeling (Figure 10.5; Lander et al. 2009). Thus, GDF11 has two major functions in feedback control of neurogenesis: to control the ratio of replicative vs. differentiative divisions of INPs, and to control the cell-cycle length of INPs.

Modeling shows that the effects of these two mechanisms on regeneration (modeled as an acute loss of most ORNs) will be profoundly different. Whereas feedback on INP division rate exerts no control over steady-state ORN numbers, feedback on the replication/differentiation choice of INPs can lead to nearly perfect control (maintaining correct ORN number independent of fluctuations in rates of ORN death, or even in numbers of stem cells or rates of stem cell division). Fikewise, the modest improvement in regeneration speed that is provided by feedback on INP division rate comes at the expense of a requirement that a very large fraction of the tissue needs to consist of INPs. As shown in Figure 10.6A, in order to drive regeneration that is threefold faster than the normal rate of ORN turnover, half the OE would need to be INPs (in reality, that number is probably less than 10%) (Smart 1971; Cuschieri and Bannister 1975; Mackay-Sim and Kittel 1991; Schwartz Fevey et al. 1991; Farbman 1992; Gordon et al. 1995; Mumm et al. 1996). In contrast, with feedback on the replication/differentiation choice of INPs, regeneration can occur up to 100% times faster than the normal rate of ORN turnover, and only a small fraction of the cells in the tissue need to be INPs (Figure 10.6B; cf. Lander et al. 2009). Moreover, regeneration under such circumstances will be characterized by a transient expansion and then contraction of the INP pool, followed by a large increase in ORN number; this is just the sort of behavior the OE displays following bulbectomy (Figure 10.1).

### 10.4.2. Multiple Feedback Loops Improve Performance

Although modeling demonstrated that GDF11, by virtue of its ability to regulate the choice between replication and differentiation by INPs, could achieve important goals of speed and robustness in the OE, further analysis revealed several problems: First, it was not possible to find conditions (numbers of cells, strengths of feedback, etc.) under which both speed and robustness could be achieved at the same time. Second, we learned that the ability to achieve explosively fast regeneration following a total loss of ORNs, only came at the expense of condemning the system to very slow regeneration following a less-than-total loss of ORNs (e.g., a 75% reduction; Figure 10.6C).

Interestingly, both of these obstacles can be overcome by introducing a second feedback loop into the system—this time directed at the behavior of the *Sox2*- and *Mash1*-expressing cells that are the progenitors of INPs (Figure 10.6D). Such cells are not responsive to GDF11, but, as mentioned earlier, respond to activins, which are also produced in the OE (Gokoffski and Calof, unpublished observations). Altogether, these findings indicate that multiple feedback loops are necessary to make the OE robust to a large range of environmental perturbations, and to permit the rapid and controlled regeneration of ORNs, which is such an important characteristic of this sensory neuroepithelium.

### 10.4.3. Follistatin (FST) Expression Creates a Stem Cell Niche in the Olfactory Epithelium (OE)

If the purpose of feedback is to report to stem and progenitor cells the overall tissue size and/or number of ORNs, then the concentrations of feedback molecules that are sensed by stem and progenitor cells need to vary proportionally (or nearly so) with tissue size and/or ORN number. Within a tissue, a secreted molecule's local concentration depends on its rate of production, diffusivity, and rate of uptake and degradation, but also on what happens to it when it reaches the boundaries of the tissue. If those boundaries are closed (i.e., the molecule cannot escape), then no matter how the tissue may grow in size, the concentration of any molecule secreted uniformly throughout the tissue will remain unchanged (this is because the volume in which the molecule is diluted goes up at the same rate as the amount of the molecule that is produced). In such a tissue, levels of secreted molecules can never provide feedback information about tissue size or numbers of terminally differentiated cells.

Few tissues are truly "closed," but epithelia are effectively closed at one end (the apical end, where tight junctions exist), and open at the other (the basal lamina), which is freely permeable to polypeptide growth factors (Dowd et al. 1999). We can calculate how the levels of secreted molecules will vary with tissue size (epithelial thickness) for such an arrangement, but only after first specifying what happens to signaling molecules after they cross the basal lamina. If we assume that they are free to wander back across into the epithelium, we get the result shown in Figure 10.7A, in which the concentration of the growth factor within the epithelium starts to plateau when the epithelium is rather small. In effect, even though the epithelium is open at one end, it behaves as if closed once it has grown beyond a certain

thickness (this thickness corresponds to about half the mean distance the signaling molecule travels within the epithelium before it is captured by receptors; this is a distance that can be estimated to be in the tens of micrometers; Lander et al. 2009). In contrast, if we specify that the growth factor is quickly and irreversibly trapped (or degraded) once it crosses the basal lamina, we get the results shown in Figure 10.7B. Now, growth factor concentration within the tissue rises over a much larger range of epithelial sizes, allowing such a growth factor to be a useful reporter of tissue size.

Such calculations are instructive because they provide a logical explanation for the localization of FST expression in the OE. As shown in Figure 10.7C, the major source of FST associated with the developing OE is in the stroma beneath the epithelium (even though genetic experiments, discussed above, show that it acts on GDF11 and activins produced within the epithelium). Since FST is known to be an irreversible binder of activins and GDF11 (Schneyer et al. 2008), it can be expected to provide just the sort of sink portrayed in Figure 10.7B, allowing such molecules to be efficient reporters of OE size (and ORN numbers).

This sort of analysis illustrates how genetics and modeling can give different, yet complementary, views of the same process. From the standpoint of genetics, FST is an inhibitor of GDF11 and activins in the OE. Modeling, however, suggests that the primary role of FST may be less to inhibit these molecules than to alter their distribution within the OE. An important consequence of this effect is to create a defined region—just above the basal lamina—where the effective concentrations of GDF11 and activins are lowest, and also vary most sensitively with epithelial size (Lander et al. 2009). Remarkably, this is precisely where stem and progenitor cells (the cells that respond to GDF11 and activins) come to reside in the OE (Figures 10.7D and E). Through its action in the stroma, FST effectively creates a stem/progenitor cell "niche" within the epithelium, where such cells are most able to proliferate, and most efficient in responding to perturbations in OE size or ORN number.

#### 10.4.4. Consequences of Feedback for Understanding Stem vs. Transit-Amplifying Cells

Recent evidence suggests that differing levels of expression of *Sox2* and *Mash1* may actually represent alternative states of a single stem/early progenitor cell, whereas *Ngn1*-expressing INPs are clearly a separate population with distinct properties and roles in development and regeneration (Murray et al. 2003; Wu et al. 2003; Beites et al. 2005; Gokoffski and Calof, unpublished observations). The fact that *Sox2/Mash1*-expressing cells give rise to INPs (which exit the cell cycle to differentiate into ORNs) might tempt classification of these as stem cells (cells that divide indefinitely and asymmetrically), and *Ngn1*-expressing cells as transit-amplifying cells (cells that are committed to a single differentiative endpoint and can only undergo limited rounds of division) (DeHamer et al. 1994; Gordon et al. 1995). However, modeling of cell lineages suggests that such behaviors are not likely to be intrinsic properties inherent to each cell population, but rather, may be characteristic behaviors of cells that occur as a consequence of feedback regulation (Lander et al. 2009). Such models of the ORN lineage and other lineages show that if stem and progenitor cells self-replicate more than half the time, then negative feedback modulation of their behaviors is sufficient to give rise to a system in which the first cell stage (*Sox2/Mash1*-expressing cells) self-replicates exactly half the time; while the second cell stage (*Ngn1*-expressing cells) undergoes an apparently limited number of cell divisions (Shen et al. 2006; Lander et al. 2009). Moreover, such modeling predicts that the "stem" cell stage can extinguish itself in such systems, which will cause the second cell stage (the "transit-amplifying cell") to adopt "stem-like" behavior. Thus, the behaviors that we think of as characterizing stem vs. transit-amplifying cell populations in regenerating tissues (Potten 1981; Jones and Watt 1993) may not be immutable, intrinsic characteristics of the cells, but rather the outcomes of these cells' responses to extrinsic signals, such as GDF11 and activin. Ultimately, these studies suggest that using cell cycle characteristics to define stem vs. transit-amplifying cells may not be the most useful means of understanding the regenerative properties of tissues (Lander et al. 2009).

## 10.5. OTHER TYPES OF FEEDBACK: INTERACTION OF TRANSFORMING GROWTH FACTOR (TGF-β)s WITH NEURAL SPECIFICATION FACTORS

#### 10.5.1. Olfactory Epithelium (OE) Formation Requires *Foxg1*

*Foxg1* (Forkhead box factor Gl, also known as Brain Factor-1; Carlsson and Mahlapuu 2002) is a homeobox transcription factor that is highly expressed in embryonic forebrain and has been reported to promote development of several anterior neural structures (Xuan et al. 1995; Hebert and McConnell 2000; Hanashima et al. 2004, 2007; Pratt et al. 2004; Martynoga et al. 2005; Pauley et al. 2006; Duggan et al. 2008). Mice null for *Foxg1* show dramatic reductions in the size of their cerebral hemispheres and are missing ventral telencephalic structures, and die shortly after birth (Xuan et al. 1995). The OE of *Foxg1*$^{-/-}$ animals is also greatly diminished or even absent, due to defects that occur early in development. In *Foxg1*$^{-/-}$ OE, cells expressing ORN lineage markers are present, but already greatly reduced in number by day 11 of gestation. As shown in Figure 10.8A, only a few *Mash1*-expressing early progenitors can be detected in a restricted domain in *Foxg1*$^{-/-}$ OE at Ell, and *Ngn1*- and Ncam-expressing INPs and ORNs are even more dramatically reduced (Kawauchi et al. 2009). OE neurogenesis and nasal cavity morphogenesis both cease early in development in *Foxg1*$^{-/-}$ mice, such that, by E13.5, *Foxg1*$^{-/-}$ mice lack an OE and most of their nasal cavity (Xuan et al. 1995; Duggan et al. 2008; Kawauchi et al. 2009).

### 10.5.2. Interaction of *Gdf11* and *Foxg1* Regulates Histogenesis and Morphogenesis of the Olfactory Epithelium (OE) and Nasal Cavity

Experiments using cultured neuroepithelial cells have demonstrated that FoxGl can bind to Smad3-containing complexes (Smad3 has been established as a component of the GDF11 cytoplasmic signaling pathway; Oh et al. 2002; Andersson et al. 2006) and thus block expression of *p21Cip1*, which encodes a cyclin-dependent kinase inhibitor that is known to be an effector of both GDF11 and TGF-β signaling (Nomura et al. 2008; Tsuchida et al. 2008). Since *p21Cip1* is also known to be expressed in the OE (Kastner et al. 2000; Legrier et al. 2001), Kawauchi and colleagues hypothesized that interactions of FoxG1 with GDF11 might be important in the regulation of OE development by FoxG1. Analysis of *Foxg1* expression in the OE using *in situ* hybridization revealed that the expression domain of *Foxg1* overlaps substantially with that of *Gdf11* throughout much of prenatal OE development (Figure 10.8B; Kawauchi et al. 2009). Transcripts of both *Gdf11* and *Foxg1* are predominantly restricted to the basal compartment of the epithelium, where stem and neuronal progenitor cells are located. However, the expression of *Foxg1* within the OE is not uniform: by E12.5, there are clear regional differences, with *Foxg1* expressed at greatest levels in the OE located in the recesses of the developing turbinates and the posterior recess of the nasal cavity (at the junction of the septum and turbinates; Figure 10.8B). These are the very regions of the OE that are most actively expanding into the nasal mesenchyme, as morphogenesis of the nasal cavity proceeds during prenatal development. In contrast, *Gdf11* expression is rather uniformly expressed within the OE, wherever OE is present in the nasal cavity (Figure 10.8B).

The presence of *Gdf11* and *Foxg1* transcripts at similar times and in the same cell populations; the known ability of FoxGl to inhibit expression of at least some GDFU target genes; and the opposite OE phenotypes that result from absence of *Gdf11* vs. absence of *Foxg1*; together raise the possibility that FoxGl regulates OE development by regulating the action of GDFU. To assess this directly, genetic epistasis experiments were performed, and the OE was analyzed in wildtype, *Foxg1*$^{-/-}$, *Gdf11*$^{+/-}$, and *Foxg1*$^{-/-}$*; Gdf11*$^{-/-}$ double mutants (Figure 10.8C and D). These experiments demonstrated that loss of *Gdf11* rescues defects in OE neurogenesis that result from inactivation of *Foxg1,* and in a *Gdf11* gene dosage-dependent manner. Figure 10.8C shows what this looks like at birth (P0): in wildtype OE, the olfactory turbinates are well-developed and the OE is easily recognized by in situ hybridization to the INP marker, *Ngn1*. In contrast, in the *Foxg1*$^{-/-}$ mice, essentially no OE is present, and there are no olfactory turbinate structures in what appears to be the vestige of the nasal cavity. However, in *Foxg1;Gdf11* double nulls, there is a significant rescue of both nasal cavity formation and OE development, and *Ngn1*-expressing cells can be observed in the basal compartment of a well-developed OE, which covers an identifiable—albeit smaller than normal—nasal cavity (Kawauchi et al. 2009).

The OE of single and double mutants, as well as *Foxg1* nulls in which only one allele of *Gdf11* was inactivated, were also examined in these studies. As shown in Figure 10.8C, cells of the ORN lineage can be easily recognized at E16.5 in wildtype animals, by their laminar positions and expression of the neuronal cell markers, *Ngn1* and *Ncam*. In contrast,

OE, nasal cavity, and ORN lineage markers are all absent in *Foxg1*$^{-/-}$ mice at E16.5. Strikingly, when *Foxg1*$^{-/-}$ embryos are also made null for *Gdf11,* both the OE and the nasal cavity itself are rescued significantly. The OE of *Foxg1*$^{-/-}$*;Gdf11*$^{-/-}$ mice is of normal thickness, and contains cells expressing major lineage markers (Figure 10.8D). Notably, when just one allele of *Gdf11* is inactivated in *Foxg1* null mutants (*Foxg1*$^{-/-}$*; Gdf11*$^{+/-}$ mice), both OE histogenesis and nasal cavity formation are significantly restored. The degree of rescue is more pronounced in double mutants compared to *Foxg1*$^{-/-}$*; Gdf11*$^{+/-}$ compound mutants, suggesting that *Foxg1*$^{-/-}$ phenotypic rescue is dependent on *Gdf11* gene dosage. In addition, the fact that removal of a single *Gdf11* allele transforms the *Foxg1*$^{-/-}$ phenotype from one in which no nasal cavity develops, into one with a nasal cavity lined by an OE of normal thickness and composition, suggests that there is a threshold level of GDF11 activity below which histogenesis and morphogenesis can proceed fairly normally, and above which these processes fail completely.

#### 10.5.3. Regulation of *Gdf11* and *Fst* Expression by *Foxg1*

As mentioned previously, the importance of FST as an endogenous antagonist of GDF11 signaling is evident by the deficits in neurogenesis observed in *Fst*$^{-/-}$ mice, in which the OE is very thin and markedly depleted of INPs and ORNs (Figure 10.4H; Wu et al. 2003). However, nasal cavity morphogenesis appears normal in *Fst*$^{-/-}$ mice, and an OE is present, although it is much thinner than normal (Figure 10.9A; Kawauchi et al. 2009). Interestingly, we found that *Foxg1*$^{-/-}$ embryos lack *Fst* expression in and around the OE from the earliest developmental stages (Figure 10.9B). This finding suggested an additional mechanism by which *Foxg1* could antagonize *Gdf11* activity: by promoting expression of *Fst, Foxg1* would lower the effective concentration of GDF11 in the OE. Consistent with this idea, when OE development in *Foxg1*$^{-/-}$ mice is rescued by removing one or more alleles of *Gdf11, Fst* expression is also restored in the tissue (Figure 10.9C). Altogether, these findings indicate that the OE phenotype in *Foxg1*$^{-/-}$ mice arises from a combination of intracellular (cell-autonomous) and extracellular (non-cell-autonomous) regulation of GDF11 signaling. This may explain why the absence of *Foxg1* leads to a more severe phenotype in the OE than that observed in *Fst*$^{-/-}$ mice.

It is worthwhile noting that control of *Fst* expression by FoxGl is unlikely to be direct. Both stromal and intraepithelial *Fst* expression are completely rescued in *Foxg1*$^{-/-}$*;Gdf11*$^{tm2/tm2}$ double mutants as well as in *Foxg1*$^{-/-}$*, Gdf11*$^{+/-}$ compound mutants (Figure 10.9C), as mentioned above. This demonstrates that neither *Foxg1* nor *Gdf11* are themselves required for *Fst* expression. Rather, these findings suggest that it is the OE that is responsible for inducing and maintaining *Fst* expression in the mesenchyme, with *Foxg1* being required to generate an OE that is competent to do so.

#### 10.5.4. *Foxg1* Antagonizes *Gdf11* Activity Directly and Indirectly

In view of the fact that FoxGl is a transcriptional regulator, we also considered the possibility that a third mechanism—a repressive effect of FoxGl on *Gdf11* expression—might also be at play in the OE. Using quantitative RT-PCR to determine *Gdf11* transcript levels in Ell.5 frontonasal tissue (this age was chosen because there is still a reasonable amount of OE remaining in *Foxg1* null animals), we found that *Gdf11* expression in *Foxg1*$^{-/-}$ and *Foxg1*$^{-/-}$*;Gdf11*$^{+/tm2}$ mutants is significantly lower than that in wildtype littermates (Kawauchi et al. 2009). This is not surprising given that *Gdf11* is expressed in the OE and there is substantially less OE tissue in such mutants than in wild-type animals. Indeed, Q-RT-PCR shows that levels of *Sox2,* a marker of OE neuroepithelial cells at this age (Figure 10.8A), are also markedly decreased in *Foxg1*$^{-/-}$ and *Foxg1*$^{-/-}$*;Gdf11*$^{+/tm2}$ mutants. However, when *Gdf11* transcript levels are normalized to *Sox2* transcript levels in the same samples, to correct for the different amounts of OE in the different mutants, it was found that *Gdf11* levels are actually two- to threefold higher, per amount of OE, in *Foxg1*$^{-/-}$ embryos than in wildtypes. This suggests that relative increases in GDF11 activity, within what little OE remains in *Foxg1*$^{-/-}$ mice, may contribute to the severity of the OE phenotype in these animals.

The observations that loss of *Foxg1* results in increased GDF11 signaling, increased *Gdf11* expression, and decreased expression of a GDF11 signaling antagonist (*Fst*), collectively suggest that the relationship between GDF11 and FoxGl activity is a highly sensitive one. If, as we suggest, it is the OE itself that induces expression of *Fst* in its underlying

stroma, then a positive feedback loop that controls OE neurogenesis emerges (Figure 10.10): an increase in *Gdf11* activity would lead to a decrease in OE size, which would cause a decrease in *Fst* expression, which would, in turn, cause an increase in *Gdf11* activity. A decrease in *Gdf11* activity would be similarly self-enhancing. According to this view, GDF11 in embryonic OE is less of a graded regulator of neuronal production than a switchlike controller of a self-sustaining program of neurogenesis—with FoxGl regulating when and where the switch is thrown (Figure 10.10).

During embryonic development of the OE, the process of neurogenesis can be viewed as serving two distinct ends: (1) histogenesis, the generation of an appropriate complement and number of OE cells at each location along the epithelium; and (2) morphogenesis, the planar growth and invagination of the epithelium that produces the deep folds characteristic of the nasal cavity. In $Foxg1^{-/-}$ embryos, both processes fail from early stages. Yet, when *Foxg1* mutants are rescued through loss of *Gdf11,* the two processes are restored to very different degrees. Histogenesis is nearly normal in $Foxg1^{-/-}$, $Gdf11^{+/tm2}$ and $Foxg1^{-/-};Gdf11^{tm2/tm2}$ mutants; but morphogenesis is impaired in $Foxg1^{-/-}$ and $Gdf11^{tm2/m2}$ mice, and even more so in $Foxg1^{-/-};Gdf11^{+/m2}$ animals (Figure 10.8C and D).

These phenotypes may be explained by the expression pattern of *Foxg1* in the developing OE: *Foxg1* is initially found throughout the OE, but soon becomes localized primarily to those areas in which planar expansion of the epithelium is occurring (Figure 10.8B). This suggests that *Gdf11* levels in most of the OE are normally low enough to permit a constant, steady accumulation of ORNs, leading to normal histogenesis. However, at locations where *Foxg1* is strongly expressed, potent inhibition of GDFl 1 signaling might allow the tissue to switch into a mode of more dramatic expansion. As discussed above, Lander and colleagues have used mathematical modeling to show that the only change needed to convert a tissue that adds cells at constant rate, to one that adds cells at an exponentially increasing rate, is adjustment of the ratio of proliferative divisions vs. differentiative divisions of a stem or transit-amplifying cell to a level above 50% (Lander et al. 2009). Since GDF11 demonstrably lowers INP replication probabilities (Figure 10.5; Wu et al. 2003; Lander et al. 2009), sufficient reduction in *Gdf11* activity could switch the OE into an exponential growth mode. However, in regions of *Foxg1* expression, GDF11 signaling is effectively blocked (through the cell-autonomous action of FoxGl on GDF11 signaling). Together, these observations explain why absence of FoxGl leads to failure of both histogenesis and morphogenesis in the OE (Figure 10.10): since unopposed GDF11 activity occurs everywhere, planar growth and neurogenesis are both halted.

## 10.6. CONCLUDING REMARKS

As with all biological systems, the key to understanding complexity is to recognize that evolution selects for that which enhances fitness. Collectively, the work summarized here demonstrates the utility of blending experimental discoveries with computational modeling: Not only are we able to uncover the key players that participate in complex biological systems, we can also gain insight as to what such complexity achieves.

Most attempts to identify and characterize molecules that regulate neurogenesis have focused on isolating factors that promote stem cell self-renewal. These efforts have been aimed at understanding how neuronal stem cell pools (which are limited in the central nervous system) might be expanded (Lennington et al. 2003; Kawauchi et al. 2005; Nystul and Spradling 2006). However, studies such as those described in this chapter, indicate that feedback—in particular, negative feedback—of self-replication and differentiation is likely to be an especially important factor in controlling the behaviors of stem and progenitor cells. Such findings indicate that identification of such negative regulators, and understanding how they function in complex systems, will be of crucial importance for advancing our basic understanding of stem cells, and for directing their eventual use in cell replacement therapies to treat injury and disease.

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## Figures

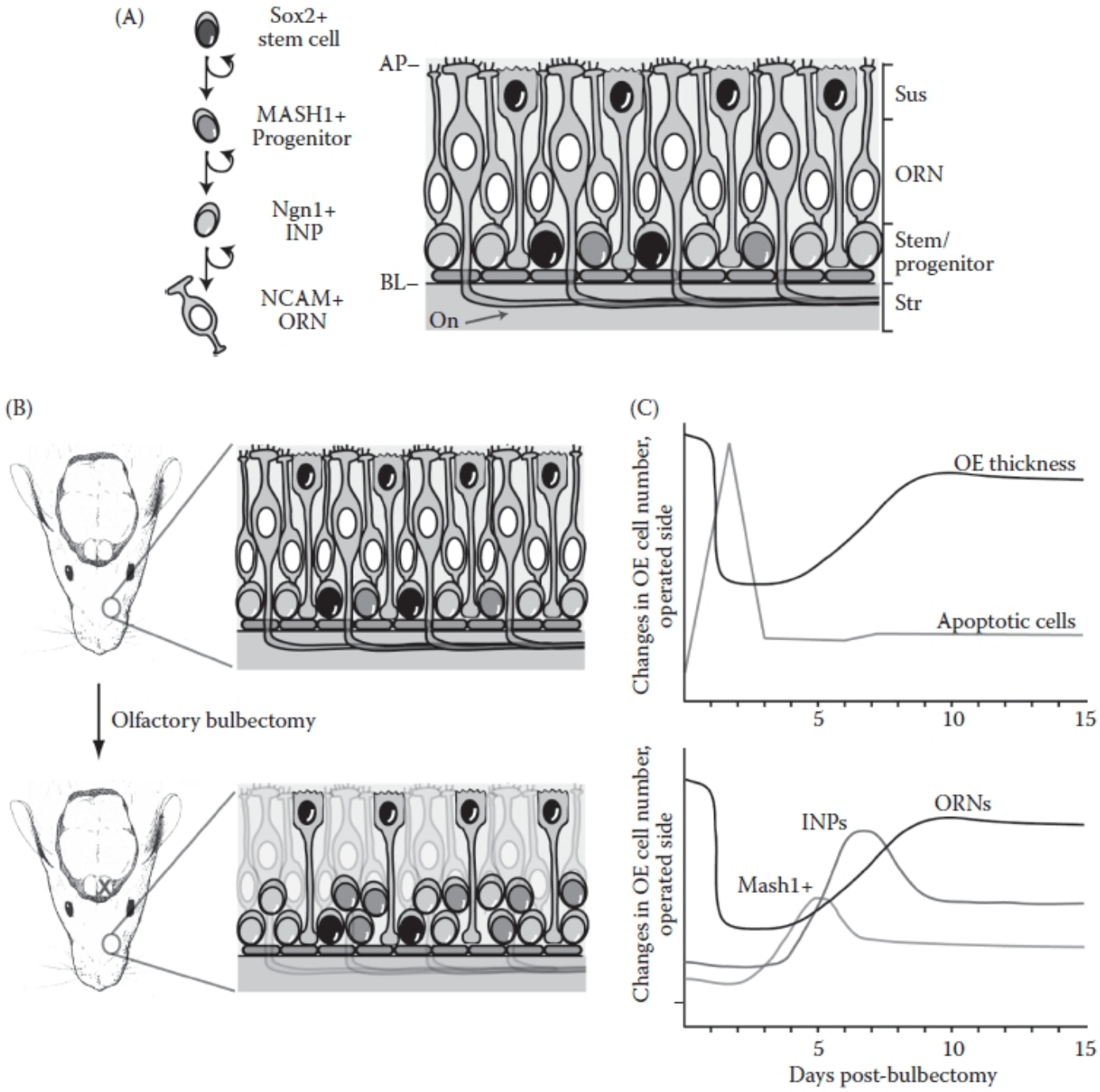



**FIGURE 10.1**

Schematic of changes in OE cell populations as a result of olfactory bulbectomy over time. (A) Diagram of ORN lineage and cell lamination in the mature OE. From apical (Ap) to basal: Sus=sustentacular cells (glial cells), with somata adjacent to the nasal cavity; ORN=olfactory receptor neuron layers, containing NCAM+ ORNs; stem/progenitor cell layers, including horizontal basal cells adjacent to the basal lamina (BL), Sox2+ stem cells, Mash 1+ early progenitor cells, and Ngn1+ immediate neuronal precursor (INP) cells; Str=stroma; On=olfactory nerve (ORN axons). (B, C) Removal of one olfactory bulb (olfactory bulbectomy) leads to rapid apoptosis of ORNs and a subsequent increase in progenitor cell numbers (MASH1+ cells followed by INPs) in the ipsilateral OE. As new ORNs are generated, progenitor cell numbers decrease until a new steady state is restored. Charts are drawn as relative changes in cell numbers in the OE ipsilateral to OB removal. (Values taken from Schwartz Levey, M., Chikaraishi, D.M., and

Kauer, J.S., *J. Neurosci.,* 11, 3556–64, 1991; Gordon, M.K. et al. *Mol. Cell Neurosci.,* 6, 363–79, 1995; Holcomb, J.D., Mumm, J.S., and Calof, A.L., *Dev. Biol.*, 172, 307–23, 1995. With permission.)

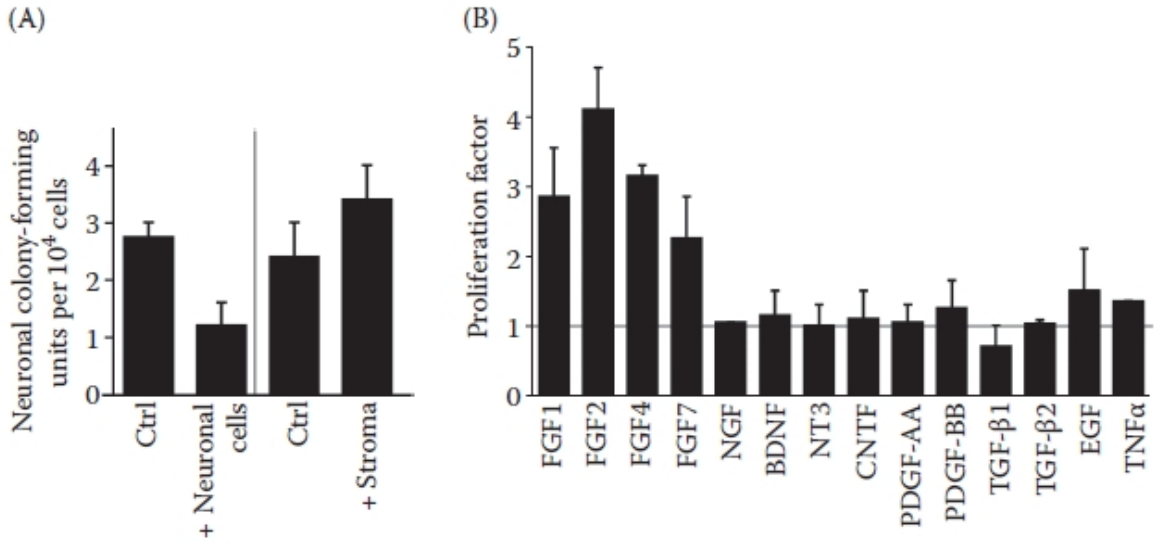
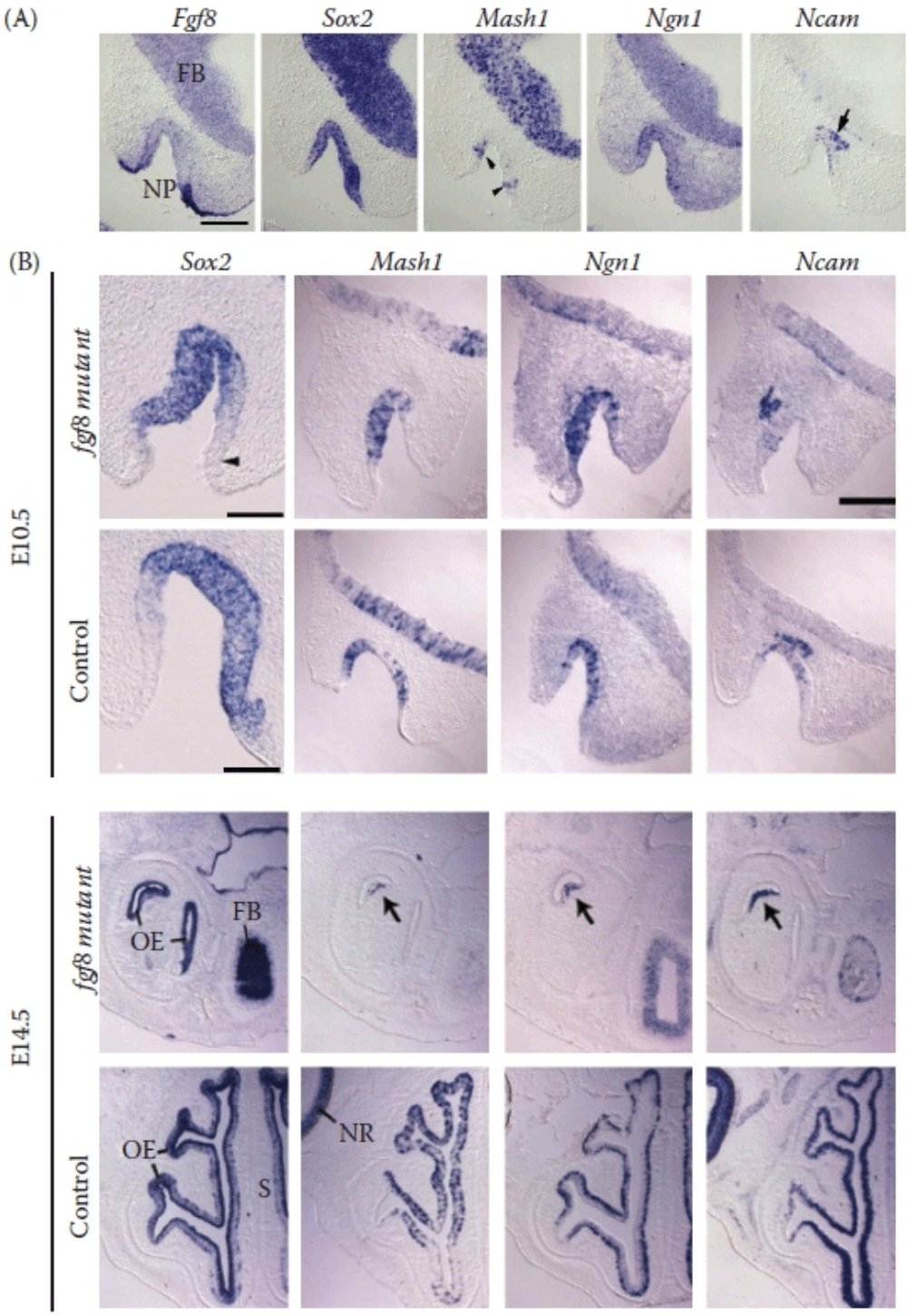
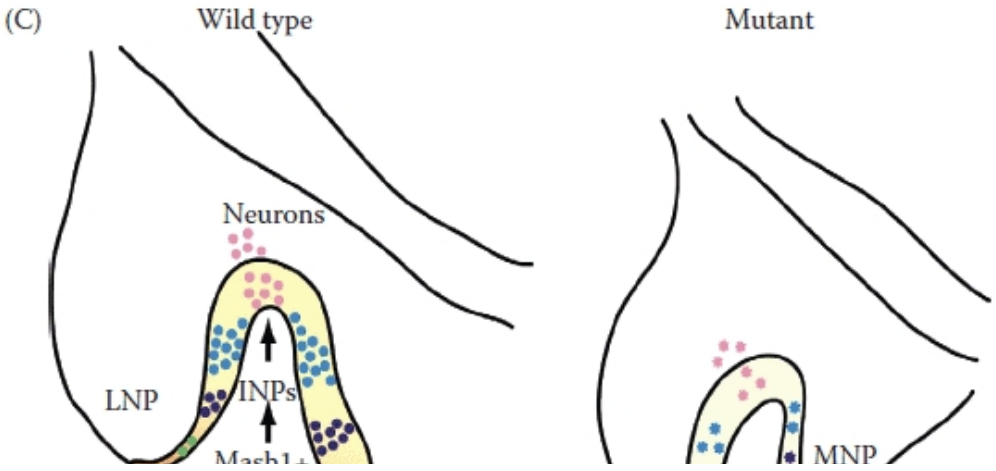



**FIGURE 10.2**

Regulators of OE neurogenesis in vitro. (A) Addition of a neuronal cell fraction containing >75% ORNs to cultures of isolated OE stem/progenitor cells inhibits neurogenesis (quantified as neuronal colony-forming units) by the stem/progenitor cells. (B) Signaling proteins that promote prolonged proliferation of INPs in vitro. Only FGFs were found to have statistically significant effects on INP proliferation. (Adapted from [A] Mumm, J.S., Shou, J., and Calof, A.L., *Proc. Natl. Acad. Sci. USA,* 93, 11167–72, 1996; [B] DeHamer, M.K. et al. *Neuron,* 13, 1083–97, 1994.)


(A)
Fgf8
Sox2
Mash1
Ngn1
Ncam
FB
NP
(B)
Sox2
Mash1
Ngn1
Ncam
E10.5
fgf8 mutant
Control
E14.5
fgf8 mutant
OE
FB
Control
OE
S
NR


(C)
Wild type
Mutant
Neurons
LNP
INPs
MNP

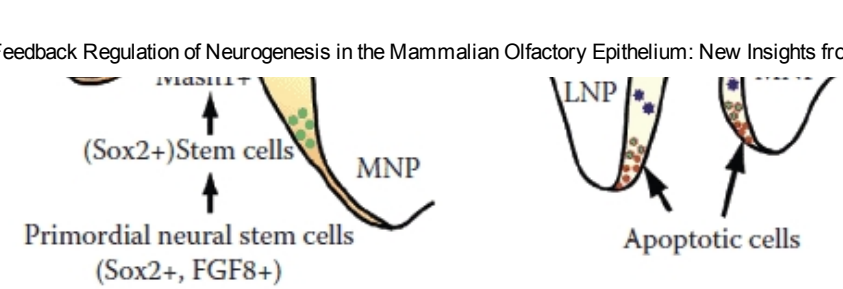



**FIGURE 10.3**

Absence of *Fgf8* leads to apoptosis of primordial *Sox2*-expressing OE neural stem cells and cessation of OE neurogenesis and nasal cavity morphogenesis. (A) Expression of *Fgf8* and neuronal lineage markers in E10.5 olfactory pit (*in situ* hybridization, ISH). Note overlap between *Fgf8* and *Sox2* expression domains. Arrowheads: *Mash1*-expressing cells; arrow: *Ncam*-expressing neurons. FB, (presumptive) forebrain; NP, nasal pit. Scale bar: 200 µm. (B) Cessation of neurogenesis in *Fgf8* mutants (conditional allele of *Fgf8* deleted using BF1-Cre) (Hebert and McConnell 2000). Arrowhead marks reduced *Sox2* expression in the OE lining the nasal pit at E10.5; arrows indicate apparent OE remnant in E14.5 mutant animals. FB, forebrain; NP, nasal pit; NR, neural retina; OE, olfactory epithelium; S, nasal septum. Scale bars: 200 µm. (C) Schematic of FGF8's role in OE neurogenesis. The sketch shows the relative positions of different neuronal cell types within the OE during primary olfactory neurogenesis at E10.5 in wildtype and *Fgf8* mutants. *Fgf8* expression domain, orange; *Sox2* expression domain (definitive neuroepithelium), yellow; *Sox2*+ stem cells, green; Mash1+ early progenitors, dark blue; *Ngn1*+ INPs, light blue; *Ncam*+ ORNs, pink. Cells in the *Fgf8*-expressing domain that undergo apoptosis when *Fgf8* is inactivated are shown in red, and apoptotic primordial neural stem cells (*Sox2*+, *Fgf8*+) are in green with red jagged border. Vestigial populations of other neuronal cell types are shown in their corresponding colors, but with jagged borders. ([C] Adapted from Kawauchi, S. et al. *Development,* 132, 5211–23, 2005.)

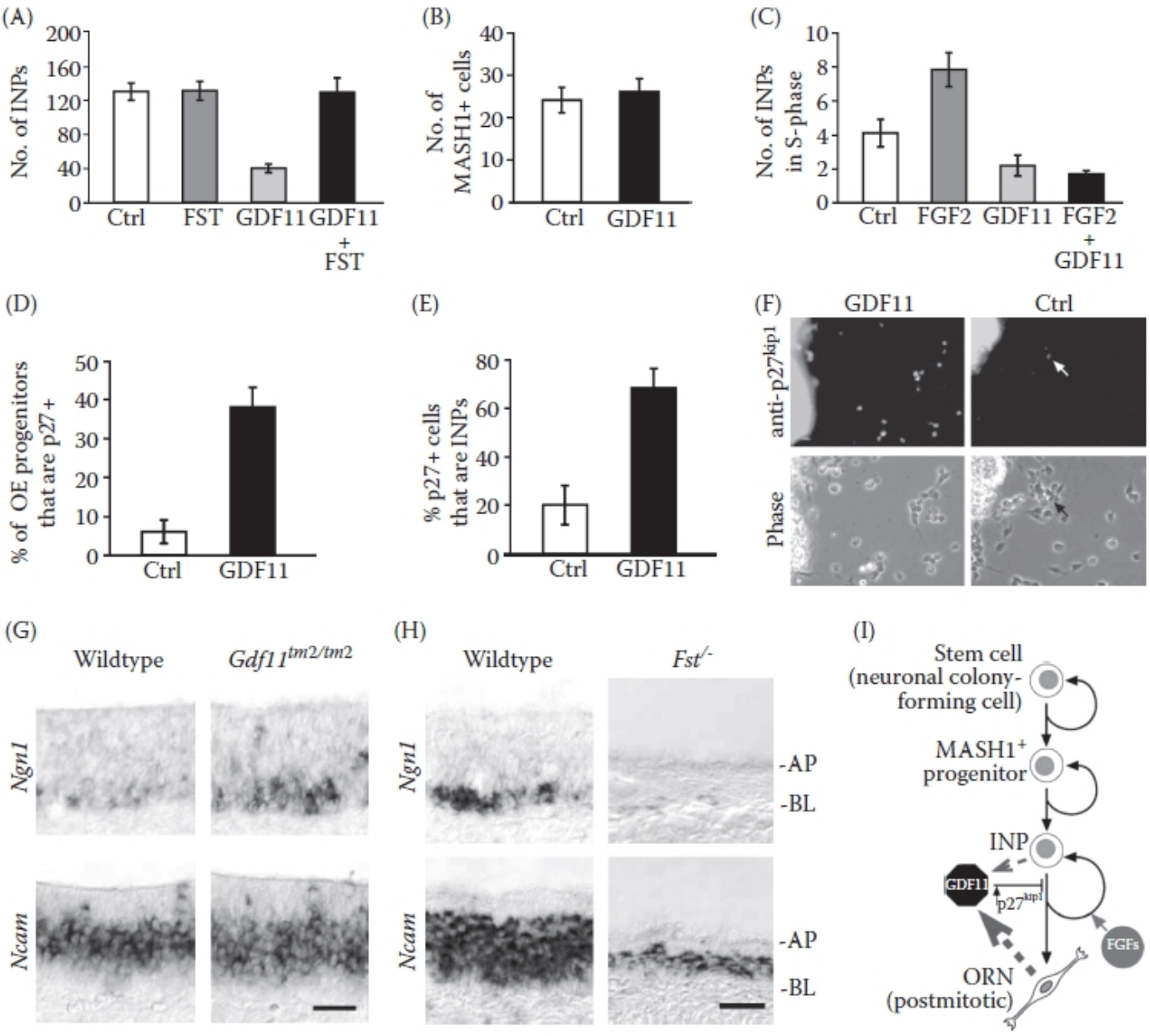



**FIGURE 10.4**

RolesofGDF11 and FST in regulating INP development and OE neurogenesis. (A) Development of INPs in culture is inhibited in the presence of GDF11. This effect is abrogated by the addition of FST. (B) GDF11 does not affect the development of MASH1+ progenitor cells. (C) GDF11 prevents FGF2-stimulated proliferation of INPs. (D, E) Many OE neuronal progenitor cells are induced to express $p27^{Kip1}$ in GDF11-treated OE cultures; most of these are INPs. (F) Addition of GDF11 induces $p27^{Kip1}$ expression in INPs in OE cultures. (G) *Gdf11*$^{tm2/tm2}$ (*Gdf11* null exhibit increased OE neurogenesis, as shown by the increase in the numbers of *Ngn1*- and *Ncam*-expressing cells in the OE. (H) Mice null for *Fst* show decreased neurogenesis, with many fewer *Ngn1*- and *Ncam*-expressing cells in the OE. (I) Schematic of GDF11 regulation of OE neurogenesis: GDF11, which is produced by INPs and ORNs, inhibits division of INPs via upregulation of $p27^{Kip1}$. Conversely, FGFs promote INP proliferation. (Adapted from Wu, H.H. et al. *Neuron,* 37, 197–207, 2003.)

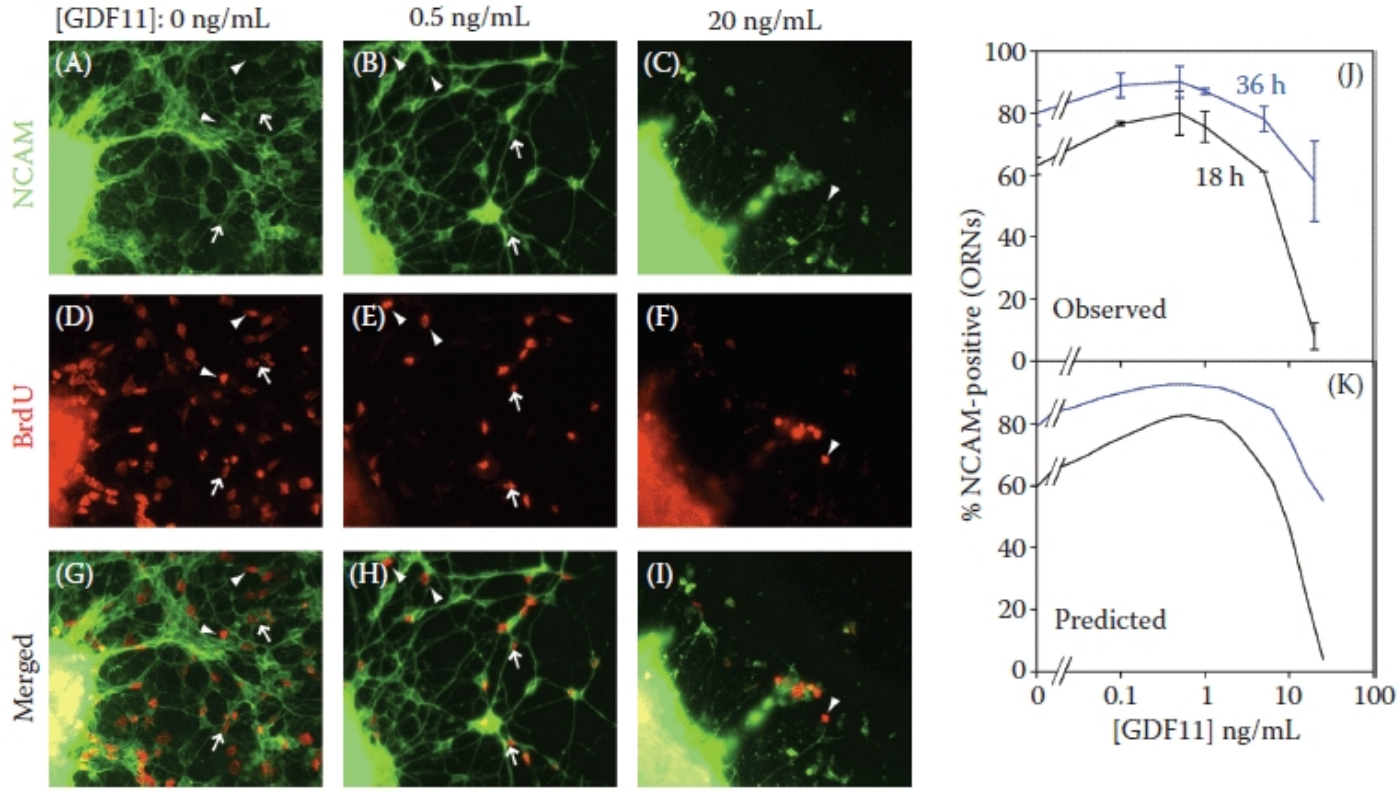



**FIGURE 10.5**

GDF11 regulates ratio of INP proliferative vs. differentiative divisions. (A–J) At low doses of GDF11, the proportion of INP progeny that differentiate into ORNs increases. At high doses, the effect reverses, with the $NCAM^{+}$ fraction falling to near zero at 18 h, but recovering at 36 h. The reversal is consistent with a slowing of the cell cycle such that 18 h is insufficient for the production of NC AM-expressing, terminally differentiated ORNs (but 36 h is). This interpretation is consistent with previous data demonstrating that high doses of GDF11 reversibly arrest the INP cell cycle (Wu et al. 2003). (K) Simulation of the experiment in (I) by a model in which GDF11 affects both ratio of proliferative vs. differentiative divisions and division rate. ([K] Reprinted from Fander, A.D. et al. *PLoS Biol.,* 7, el5, 2009.)

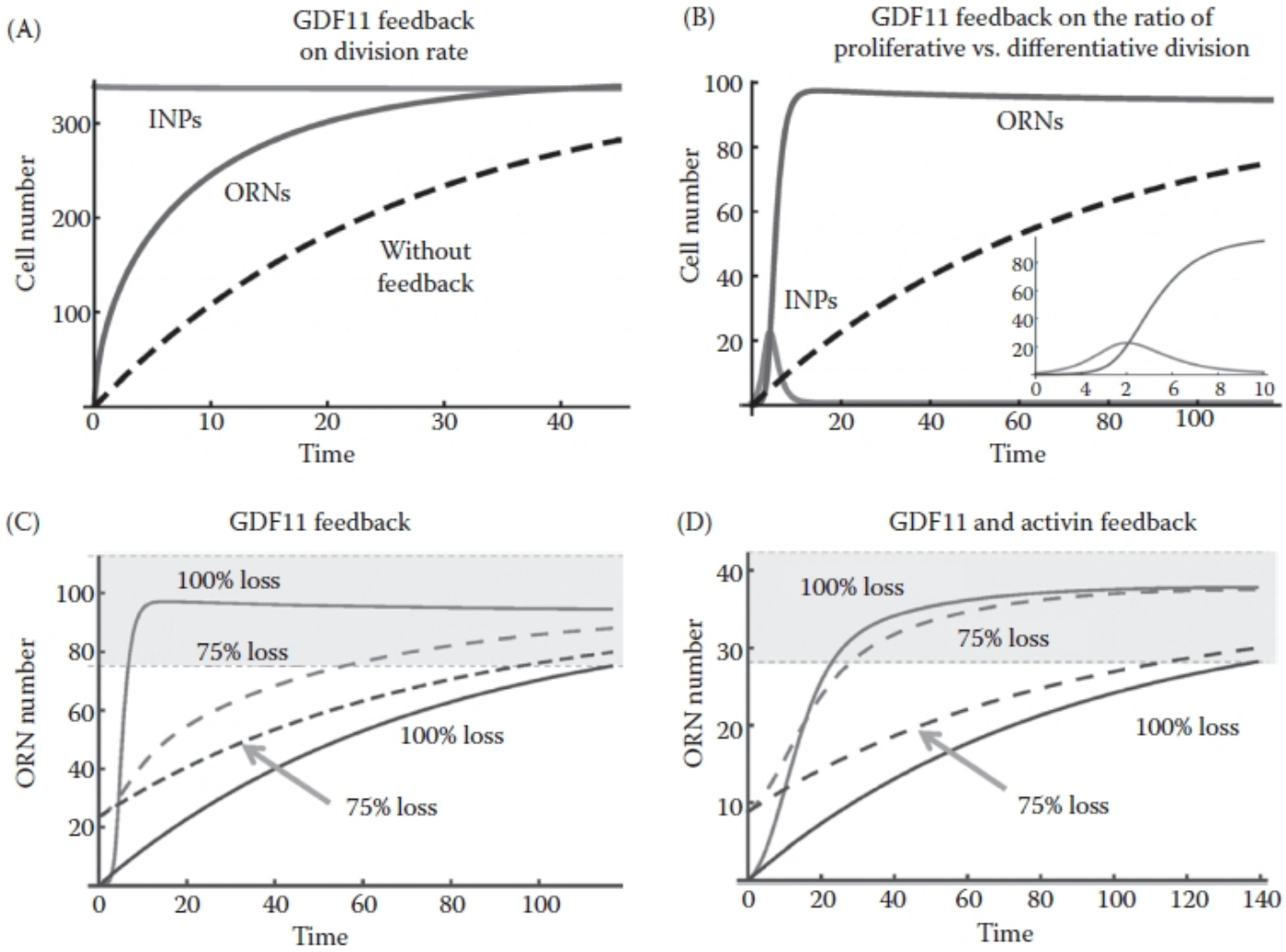



**FIGURE 10.6**

Strategies for feedback regulation of transit-amplifying cells. (A) Simulated return to steady state, after removal of all ORNs, of a system with negative feedback regulation on the INP cell-cycle length (i.e., division rate). Note that feedback leads to modestly improved regeneration speed (over what would occur in the absence of feedback; dashed line), but only when INP numbers are almost as high as those of ORNs. (B) Simulated return to steady state, after removal of all ORNs, of a system with negative feedback regulation of the ratio of INP proliferative vs. differentiative divisions. Note the much greater improvement in regeneration speed (over absence of feedback; dashed line) without necessitating a high INP reserve. Inset shows response at early times in greater detail. (C) Dependence of rate of regeneration on the severity of initial ORN depletion, for the case shown in (B). Notice how the rate of return to steady state after a partial (75%) ORN loss (dashed gray curve) is only slightly better than in the absence of feedback (dashed black curve). (D) Simulated regeneration experiment similar to that in (C), except that both GDF11 and activin feedback loops are included in the model. Now, regeneration following 75% ORN depletion is almost as fast as from 100% depletion (compare with [C]). (Adapted from Lander, A.D. et al. *PLoS Biol.,* 7, el5, 2009.)

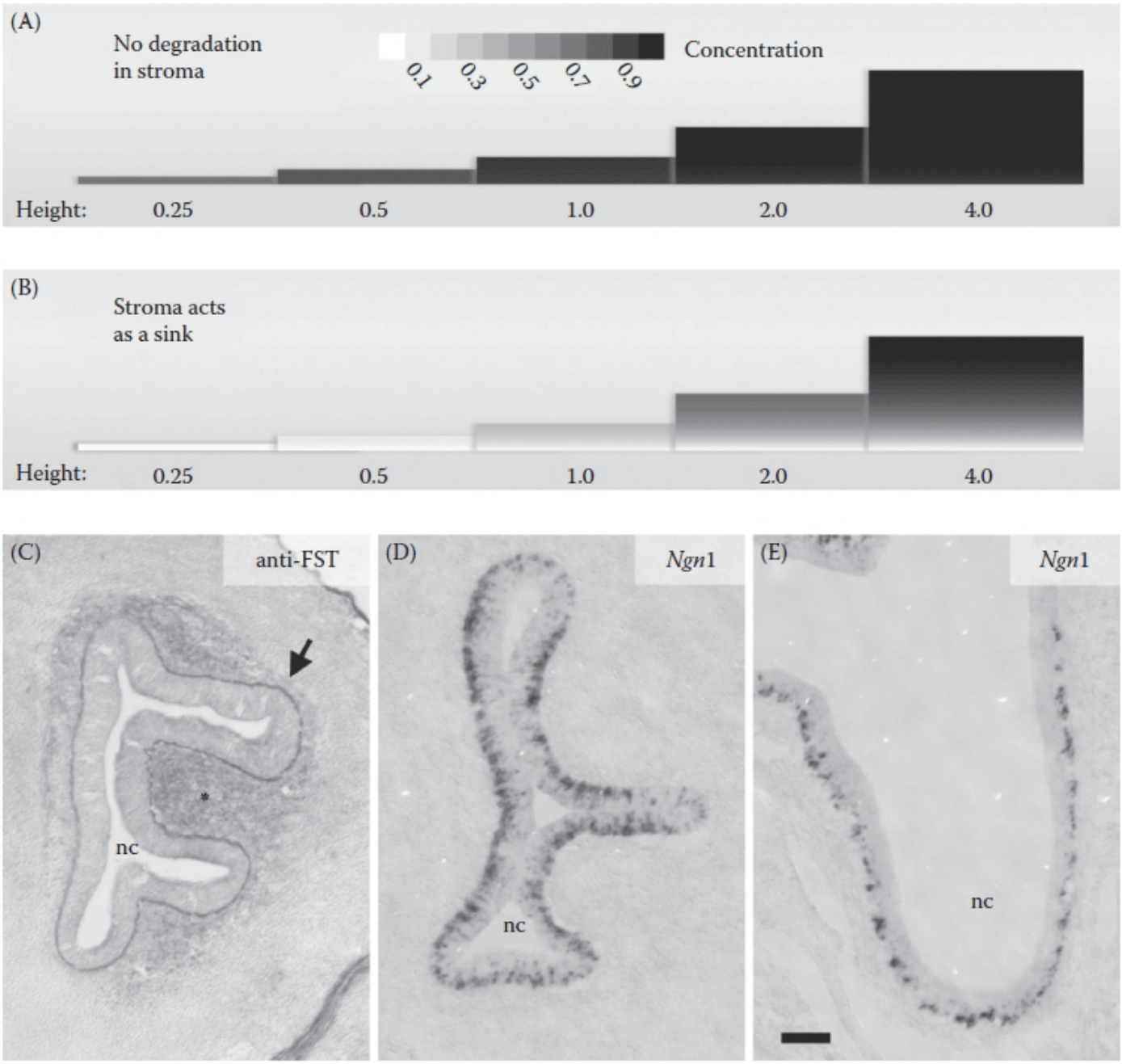



**FIGURE 10.7**

Effects of geometry and degradation on levels of secreted molecules within epithelia. (A, B) Two processes remove polypeptides secreted into the intercellular space of an epithelium: diffusion into underlying connective tissue (stroma) and degradation within the epithelium. Given a molecule's rate of production, its diffusivity, its rate of uptake and degradation, and the geometry of the epithelium, one may calculate its steady-state distribution. Here, such calculations are shown graphically, for epithelia of different thicknesses (in each picture the epithelium is oriented with the apical surface at the top). Epithelial thickness ("height") is scaled according to the decay length of the molecule of interest. The shading in each picture depicts the concentration of the secreted molecule, with black representing the limiting concentration that would be achieved in an epithelium of infinite thickness. (A) The degradation capacity of the stroma is set at one-tenth that in the epithelium. In this case, intraepithelial concentrations of secreted molecules plateau while the epithelium is very thin. (B) The stroma is treated as a strong sink, i.e., few molecules that enter it escape undegraded. Now there is a large (and more physiological) range of epithelial thickness over which the concentrations of secreted molecules grow appreciably with tissue size. This is particularly true near the basal surface of the epithelium. (C) Follistatin (FST), a molecule that binds GDF11 and activin essentially irreversibly, is present at high levels in the basal

lamina (arrow) and stroma (asterisk) at El 3 OE. Size bar: 100 urn. (D, E) INPs (visualized with *Ngn1* in situ hybridization) become progressively localized to the basal surface of the OE over the course of development. (D)=E12.5; (E)=E18.5. nc=nasal cavity. Size bar: 100 pi. (Adapted from Lander, A.D. et al. *PLoS Biol.,* 7, el5, 2009.)

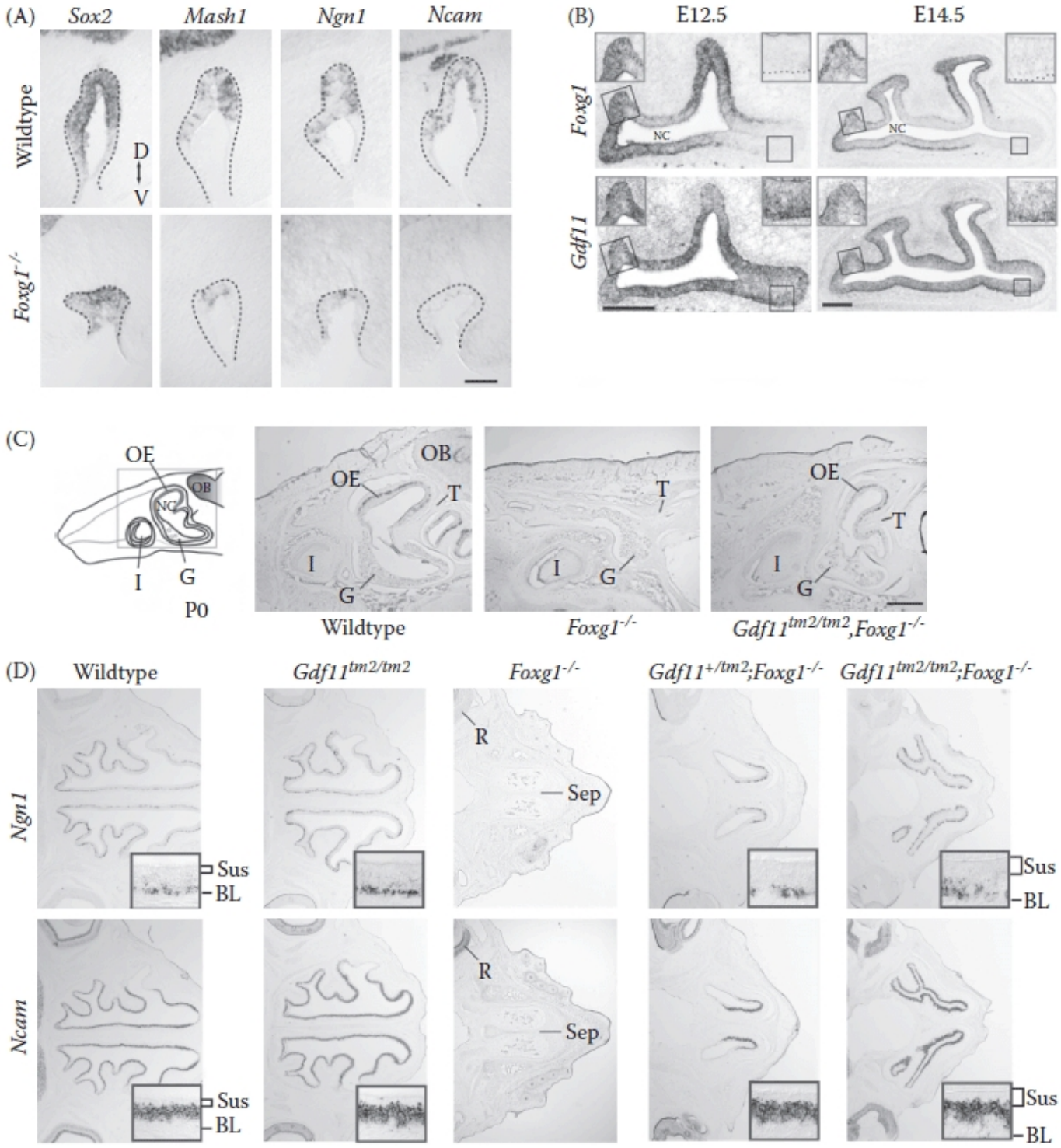



**FIGURE 10.8**

Absence of *Gdf11* rescues deficits in neurogenesis and morphogenesis observed in *Foxg1*$^{-/-}$ OE. (A) Failure of neurogenesis in *Foxg1*$^{-/-}$ OE. Sections of OE from wildtype and *Foxg1*$^{-/-}$ embryos at E11 show that olfactory pits are greatly reduced in size. The total area of *Sox2*-expressing neuroepithelium is also reduced in the mutant compared to wildtype. Only a few *Mash1*$^{+}$ early progenitors can be detected, and the decrease in *Ngn1*-expressing INPs and *Ncam*-expressing ORNs is even more dramatic. D, dorsal; V, ventral. Scale bar: 100 µm. (B) Expression of *Foxg1* and *Gdf11* in developing mouse OE. Horizontal sections show the OE in one-half of the nasal region (septum is at bottom) at E12.5 and E14.5 in wildtype mice (anterior is right, posterior is left). Expression of *Foxg1* and *Gdf11* overlap except in anterior OE, which has ceased planar expansion at these ages. Insets show high magnification of the OE at posterior regions of coexpression and anterior regions where coexpression has ceased. Dotted line indicates basal lamina. NC,

nasal cavity; scale bars: 200 μm. (C) Rescue of *Foxg1*$^{-/-}$ OE phenotype by loss of *Gdf11*. The sketch is of a midsagittal section through the frontonasal structure of wildtype P0 mice. Box indicates region of images on left. Images show *Ngn1* expression in the OE neuroepithelium where it contains neuronal progenitor cells. Olfactory turbinate structures and *Ngn1*-expressing INPs are not observed in *Foxg1* mutants; mice that are null for *Gdf11* as well as *Foxg1* (*Gdf11*$^{tm2/tm2}$; *Foxg1*$^{-/-}$ mice) show recovery of turbinate structures and OE. G, serous gland; I, incisor tooth; OE, olfactory epithelium; OB, olfactory bulb; NC, nasal cavity; T, turbinate bone. (D) Rescue of OE neurogenesis in *Foxg1*$^{-/-}$ is dependent on *Gdf11* gene dosage. ISH for OE neuronal lineage markers (*Ngn1* and *Ncam*) in the OE of E16.5 wildtype and mutant littermates. Insets show high magnification views of septal OE. In *Gdf11*$^{tm2/tm2}$ mice, *Ngn1*- and *Ncam*-expressing cell layers (and hence overall OE) are thicker compared to wildtype, as reported previously (Wu et al. 2003). No discernable OE structure is evident in *Foxg1*$^{-/-}$ mice at the same dorsoventral level. Loss of one allele of *Gdf11* (*Gdf11*$^{+/tm2}$; *Foxg1*$^{-/-}$) rescues all cell types in the OE, and the OE appears of normal thickness, although planar expansion of the OE and morphogenesis of the nasal cavity are clearly deficient in the compound mutant. Rescue is more pronounced in double nulls (*Gdf11*$^{tm2/tm2}$; *Foxg1*$^{-/-}$), particularly in terms of OE planar expansion and nasal cavity morphogenesis. For all panels, posterior is left, anterior is right; scale bar: 400 μm. (Adapted from Kawauchi, S. et al. *Development,* 2009.)

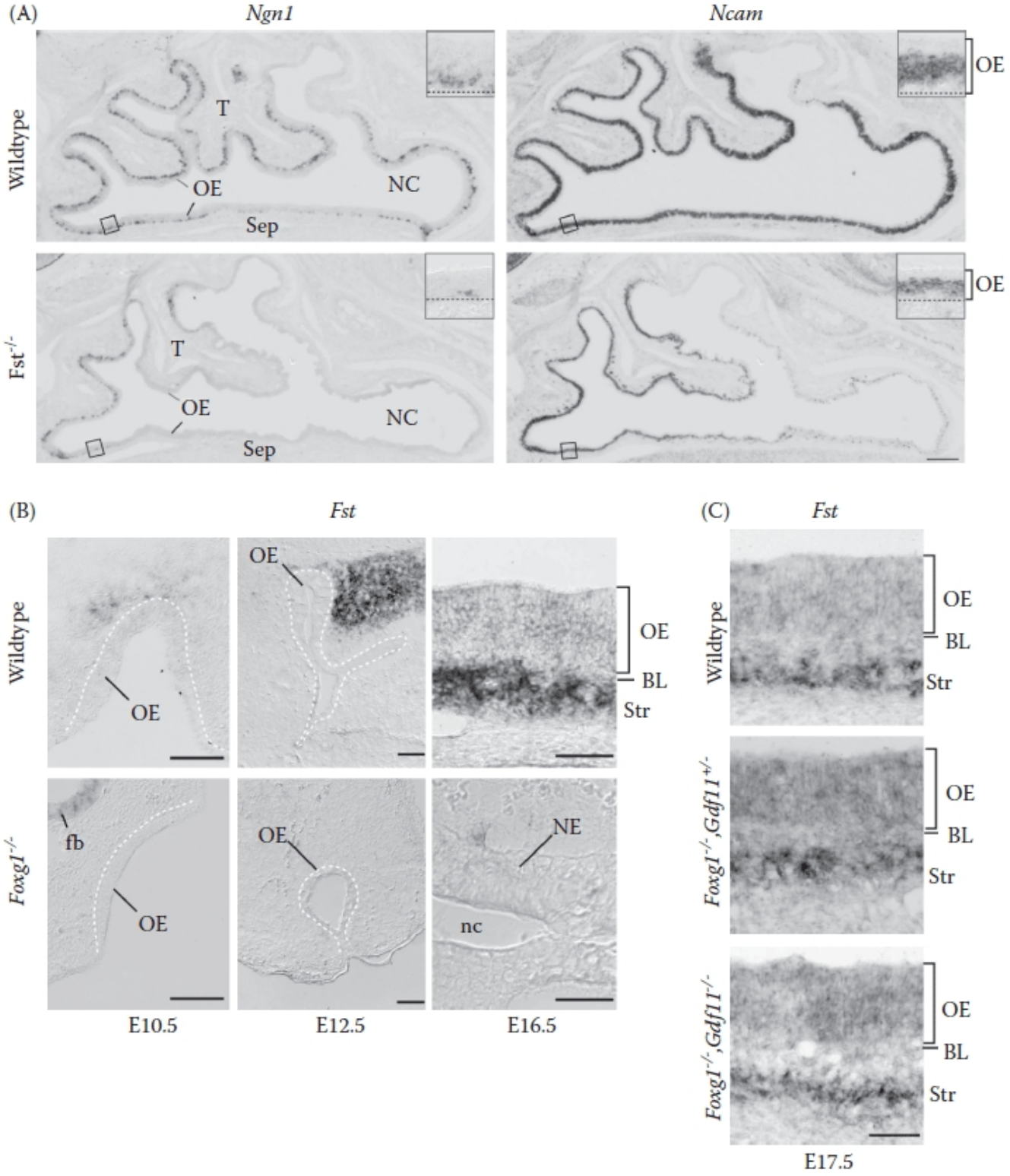



**FIGURE 10.9**

Loss of *Fst* expression in *Foxg1*$^{-/-}$ OE is rescued by inactivation of *Gdf11*. (A) Defective OE neurogenesis (histogenesis), but not nasal cavity morphogenesis, is observed in *Fst*$^{-/-}$ olfactory epithelium. ISH for OE neuronal lineage markers (*Ngn1* and *Ncam*) was performed on the OE of E17.5 wildtype and *Fst*$^{-/-}$ mutant littermates. In *Fst*$^{-/-}$ mice, the basic size and shape of the nasal cavity are the same as in wildtype, and convoluted turbinate structures are observed in the mutants. The OE of *Fst*$^{-/-}$ mice is, however, much thinner than that of wildtype littermates and is relatively devoid of *Ngn1*-expressing INPs, with a much thinner *Ncam*-expressing ORN layer (see insets). Anterior is right, posterior is left, lateral is top, the nasal septum is at the bottom of each panel. NC, nasal cavity; T, turbinate; OE,

olfactory epithelium; sep, septum; scale bar: 200 μm. (B) ISH for *Fst* performed on wildtype and $Foxg1^{-/-}$ mice at different developmental stages. At E16.5, when *Fst* is expressed in both the OE and underlying stroma in wildtype mice (B, top right panel), it is undetectable anywhere in the nasal mucosa of $Foxg1^{-/-}$ embryos (in those rare instances when remnants of nasal mucosa can be observed). NE, nasal epithelium; OE, olfactory epithelium; BL, basal lamina; nc, nasal cavity; Str, stroma; fb, forebrain; scale bars: 100 μm in E10.5 and E12.5, 50 μm in E16.5. (C) *Fst* expression is restored in rescued OE (and underlying stroma) of $Gdf11^{+/-};Foxg1^{-/-}$ and $Gdf11^{-/-};Foxg1^{-/-}$ mice ($Gdf11^{-}$ is used to designate the $Gdf11^{tm2}$ allele in this figure). Scale bar: 50 μm. (Adapted from Kawauchi, S. et al. *Development,* 2009.)

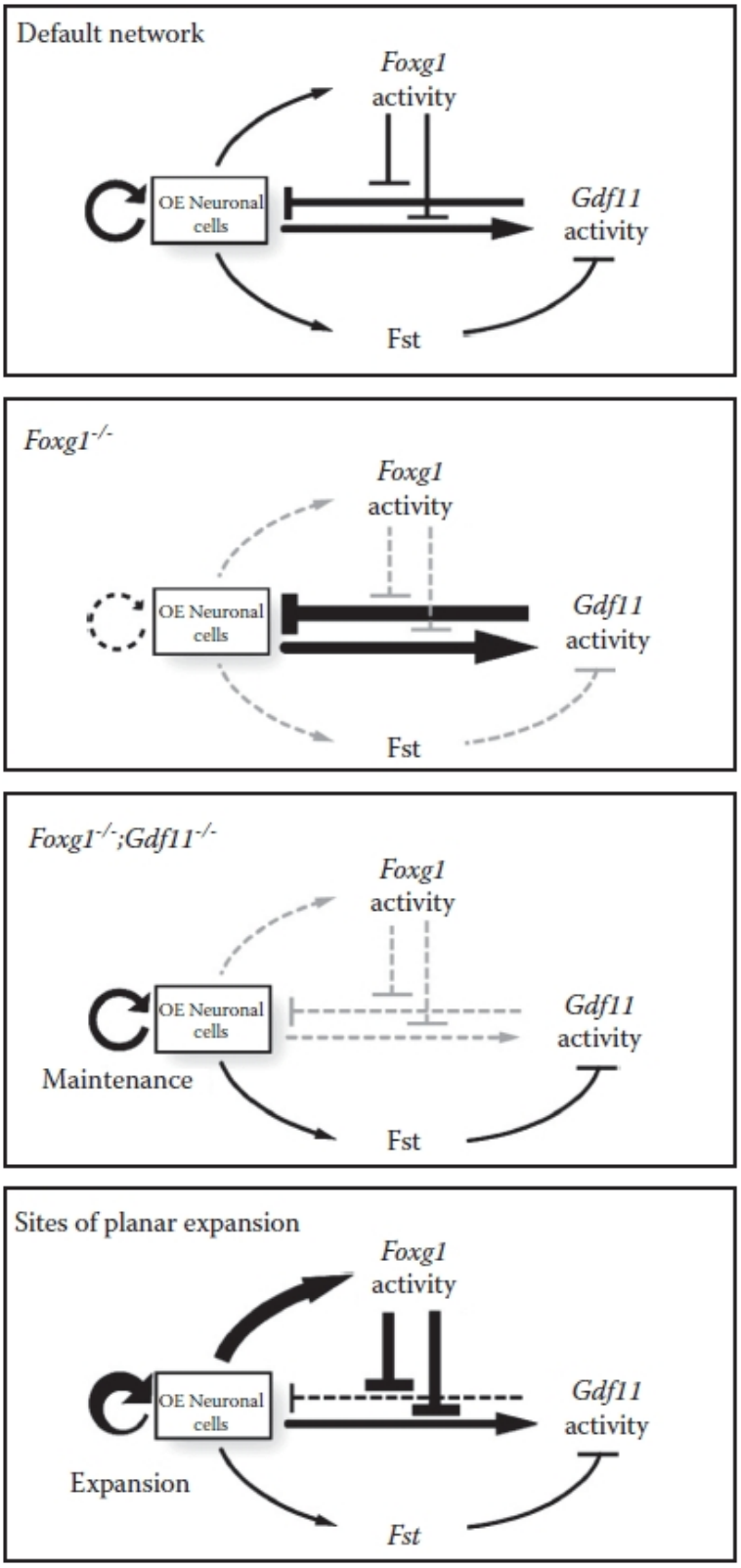



**FIGURE 10.10**

Schematic model of *Foxg1-Gdf11* interactions controlling OE neurogenesis. Default network: in wildtype OE, *Foxg1* and *Gdf11* are both produced by OE neuronal cells, but *Foxg1* proneurogenic activity antagonizes both the antineurogenic activity of *Gdf11,* and the production of *Gdf11* by OE neuronal cells. OE neuronal cells also express *Fst,* and *Fst* action antagonizes *Gdf11* activity. This default network of gene activities controls the normal steady-state level of neurogenesis in the OE. *Foxg1*$^{-/-}$ OE: In *Foxg1*$^{-/-}$ OE, *Foxg1* activity is absent, and *Fst* expression is downregulated, resulting in hypersensitivity of the OE to the action of *Gdf11.* Both OE neurogenesis and planar

expansion of the OE fail. *Foxg1*$^{-/-}$*;Gdf11*$^{-/-}$ double mutant OE: *Fst* expression is restored and histogenesis (neurogenesis) within the OE is rescued, since the antineurogenic activity of *Gdf11* is now removed and any similar antineurogenic factors are antagonized by *Fst.* Sites of planar expansion: *Foxg1* activity strongly inhibits both *Gdf11* activity and expression, which would allow the OE to undergo planar expansion in sites where *Foxg1* is highly expressed in wildtype OE (e.g., posterior recess of the nasal cavity). Once expansive growth is finished, *Foxg1* expression is downregulated (e.g., anterior septum), and OE neurogenesis returns to its default state. (Adapted from Kawauchi, S. et al. *Development,* 2009.)

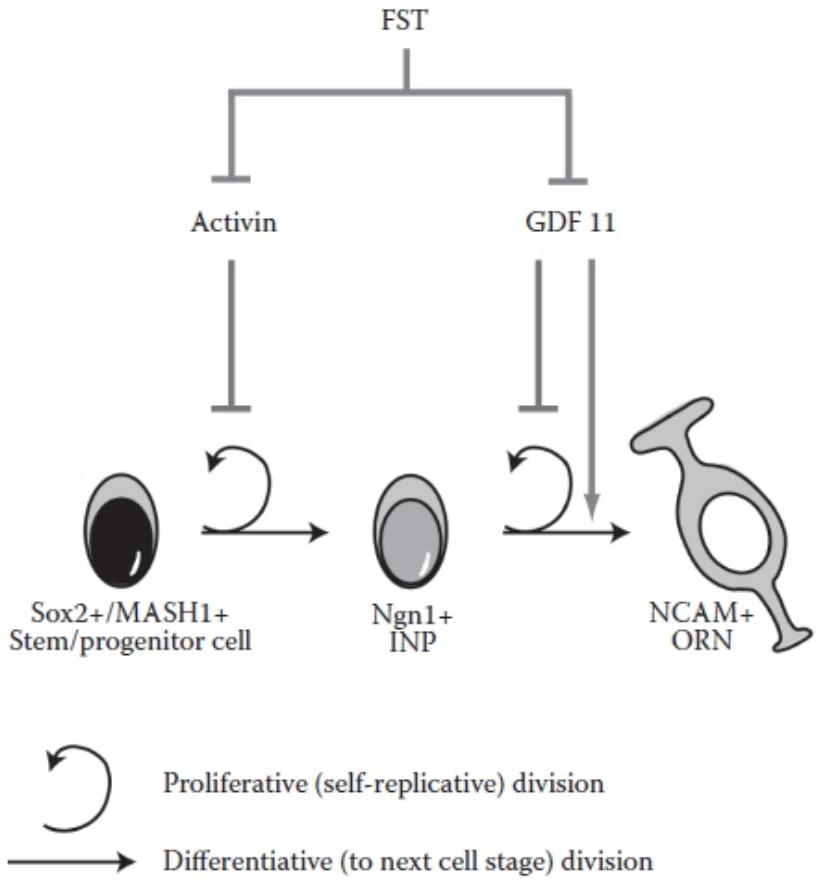



**FIGURE 10.11**

Schematic of feedback regulation of the ORN lineage. ORN production is the result of stem and progenitor cell divisions that are replicative (curved arrows) and differentiative (straight arrows). GDF11, which is produced by INPs and immature ORNs, negatively regulates replicative divisions of INPs and promotes differentiation of ORNs from INPs (Adapted from Wu, H.H. et al. *Neuron,* 37, 197–207, 2003; Lander, A.D. et al. *PLoS Biol*, 7, el5, 2009). Activin produced in the OE negatively regulates replicative divisions of *Sox2*- and *Mash1*-expressing stem/early progenitor cells (Gokoffski and Calof, unpublished observations). FST, which is synthesized in both OE and underlying stroma, antagonizes activin and GDF11 signaling (Adapted from Wu, H.H. et al. *Neuron,* 37, 197–207, 2003; Gokoffski and Calof, unpublished observations).



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