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Activin and GDF11 collaborate in feedback control of neuroepithelial stem cell proliferation and fate

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SUMMARY

Studies of the olfactory epithelium model system have demonstrated that production of neurons is regulated by negative feedback. Previously, we showed that a locally produced signal, the TGF β superfamily ligand GDF11, regulates the genesis of olfactory receptor neurons by inhibiting proliferation of the immediate neuronal precursors (INPs) that give rise to them. GDF11 is antagonized by follistatin (FST), which is also produced locally. Here, we show that *Fst*^{-/-} mice exhibit dramatically decreased neurogenesis, a phenotype that can only be partially explained by increased GDF11 activity. Instead, a second FST-binding factor, activin β (ACT β B), inhibits neurogenesis by a distinct mechanism: whereas GDF11 inhibits expansion of INPs, ACT β B inhibits expansion of stem and early progenitor cells. We present data supporting the concept that these latter cells, previously considered two distinct types, constitute a dynamic stem/progenitor population in which individual cells alternate expression of *Sox2* and/or *Ascl1*. In addition, we demonstrate that interplay between ACT β B and GDF11 determines whether stem/progenitor cells adopt a glial versus neuronal fate. Altogether, the data indicate that the transition between stem cells and committed progenitors is neither sharp nor irreversible and that GDF11, ACT β B and FST are crucial components of a circuit that controls both total cell number and the ratio of neuronal versus glial cells in this system. Thus, our findings demonstrate a close connection between the signals involved in the control of tissue size and those that regulate the proportions of different cell types.

KEY WORDS: TGF β , Olfactory epithelium, Transit-amplifying cell, Neurogenesis, Follistatin, Gliogenesis, Mouse

INTRODUCTION

For developing tissues to attain their proper size and cell type proportions, stem cell proliferation and differentiation must be tightly regulated. Recent studies have identified a variety of negative-feedback strategies for achieving robust control of tissue size and cell number (Shraiman, 2005; Kirouac et al., 2009; Lander et al., 2009). The olfactory epithelium (OE) of the mouse has been a particularly useful model system in this regard (Gokoffski et al., 2009).

The OE contains two differentiated cell types: olfactory receptor neurons (ORNs), which relay olfactory information to the central nervous system, and sustentacular (Sus) cells, which are glial cells that form the apical surface monolayer of the OE (Farbman, 1992). A multistage lineage underlies the production of ORNs (Fig. 1A) and includes: (1) stem cells, which express *Sox2* and are crucial for morphogenesis of the OE and nasal cavity (Kawauchi et al., 2005;

Kawauchi et al., 2009); (2) *Ascl1* (*Mash1*)-expressing early progenitors, which evidence indicates are committed to neuronal differentiation and required for ORN production (Guillemot et al., 1993; Gordon et al., 1995; Murray et al., 2003); and (3) immediate neuronal precursors (INPs), which express *Neurog1* (*Ngn1*) and can either self-renew or differentiate into (4) post-mitotic, *Ncam* (*Ncam1*)-expressing ORNs (Calof and Chikaraishi, 1989; DeHamer et al., 1994; Lander et al., 2009). Much less is known about the origins of Sus cells (Matulionis, 1976; Huard et al., 1998; Murray et al., 2003).

ORN number is regulated by the TGF β ligand GDF11, which when absent leads to the production of supernumerary INPs and ORNs. GDF11 has two effects: it inhibits cell-cycle progression of INPs and it decreases the proportion of INPs that self-renew rather than differentiate into ORNs (Wu et al., 2003; Lander et al., 2009). Thus, GDF11 serves as an autocrine negative growth regulator or ‘chalone’, much like its close homolog GDF8 (myostatin) in muscle (Gamer et al., 2003). Additional evidence that GDF11 is a negative regulator of OE neurogenesis comes from studies of follistatin (FST), a GDF11 antagonist: the OE of *Fst*^{-/-} mice is abnormally thin, with markedly fewer INPs and ORNs (Wu et al., 2003).

There are reasons to suspect that GDF11 is not the sole regulator of cell number and tissue size in the OE. For example, computational modeling suggests that additional feedback control must be exerted on stem cells, rather than just on the committed progenitor (INP) upon which GDF11 acts (Lander et al., 2009). As described below, our finding that the phenotype of the *Fst*^{-/-} OE is only partially rescued by loss of *Gdf11* led us to identify activin β (ACT β B; also known as inhibin β -B – Mouse Genome informatics) as a second feedback factor in the OE, and one that specifically targets *Sox2*- and *Ascl1*-expressing stem and early progenitor cells. We show here that ACT β B and GDF11 together control not only the size of the stem

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and progenitor cell populations, but also influence the choice between neuronal and glial fates assumed by these cells. We argue that this choice occurs through resolution of an initial state in which a bipotential stem cell alternates expression of *Sox2* and/or *Ascl1*. Thus, the molecular circuitry underlying OE growth regulation involves feedback effects on fate choice, proliferation and differentiation at all lineage stages.

MATERIALS AND METHODS

Reagents

GDF11 and BMP2 were from Dr Anthony Celeste (Wyeth). Activin B and FGF2 were from PeproTech. FST was from the National Hormone & Pituitary Program (A. F. Parlow, Harbor-UCLA). Bromodeoxyuridine (BrdU) for tissue culture was from Amersham (#RPN201) and for in vivo labeling was from Sigma (#B-5002). MG132 was from Calbiochem (#474790).

Animals

CD-1, *Ascl1*^{+/-}, *TgNI-2G*^{+/+}, *Fst*^{+/-} and *Gdf11*^{+/-} (*Gdf11*^{+/m2}) mice were maintained as described (Wu et al., 2003). *Ascl1*^{GFP/+} (Kim et al., 2007) and *ActfβB*^{+/-} mice (Vassalli et al., 1994) were maintained on C57BL/6J (Jackson Labs). *Ascl1*^{CreERT2/+} mice were generated as described (Kim et al., 2011). For lineage tracing, *Ascl1*^{CreERT2/+} mice were crossed with R26R-stop-YFP mice (Jackson Labs), and pregnant dams were injected with tamoxifen (Tmx; 2.5 mg/40 g body weight; Sigma, T55648) in sunflower oil (Kim et al., 2007).

In situ hybridization (ISH) and immunofluorescence (IF)

ISH and IF were performed on E17.5 OE cryosections as described (Wu et al., 2003; Kawauchi et al., 2009). New probes were *Alk4* (31-567 bp of GenBank #Z31663); *Actr11a* (*Acvr2a*; 71-472 bp of GenBank #M65287); *ActfβB* (1-602 bp of GenBank #X83376 plus 234 bp of 5' sequence); activin βA (*ActfβA*; 1049-1474 bp of GenBank #NM008380 including 50 bp of pro-domain and 24 bp of 3'UTR); and *Sox2* (1281-2029 bp of GenBank #X94127). Primary antibodies were rabbit anti-SOX2 (Chemicon, 1:500), rabbit anti-GFP (Molecular Probes, 1:500), mouse anti-cytokeratin 18 (Millipore RGE53, 1:50) and OE SUS cell-specific mouse monoclonal antibody SUS-4 (Goldstein and Schwob, 1996) (1:50).

BrdU and ASCL1 IF were performed as described (Wu et al., 2003). For CYT18/SOX2 IF, tissue was fixed in Omnifix (AnCon Genetics). Secondary antibodies were from Jackson ImmunoResearch (1:50-1:100) and nuclei were stained with Hoechst 33258 (Sigma; 10 μg/ml). Samples were visualized with a Zeiss Axiophot and/or ApoTome epifluorescence microscope, and measurements made using AxioVision software (Zeiss). For quantification, 2-5 mm of septal OE were evaluated in two to three animals of each genotype.

Primary OE cultures

To analyze ASCL1⁺ progenitors, 8-hour OE explants were prepared from CD-1 embryos, ASCL1⁺ IF performed and cells quantified as described (Gordon et al., 1995). To assess effects on INPs, explants from *TgNI-2G*^{+/-} embryos were cultured for 30 hours. The only progenitors present in OE cultures at 30 hours in vitro are INPs, which in *TgNI-2G*^{+/-}-derived OE explants are marked by expression of a *Neurog1*-specific GFP reporter (Wu et al., 2003). Sus cell cultures included FGF2 (10 ng/ml) as well as the indicated factors, and were fixed in acetone (DeHamer et al., 1994) for CYT18 IF. Migratory cells with CYT18 fluorescence intensities greater than 2.2-fold background (no primary antibody) were designated Sus. For quantification of results, at least 2000 migratory cells were counted in a minimum of three separate cultures for each condition.

RESULTS

GDF11 alone does not account for all negative-feedback signaling in the OE

Fig. 1A illustrates OE structure and the cell lineages underlying ORN production, including feedback from ORNs onto INPs by GDF11 (Wu et al., 2003; Lander et al., 2009). FST, a known

GDF11 antagonist (Schneyer et al., 2008), is also expressed in olfactory mucosa, and we have reported previously that *Fst*^{-/-} OE is deficient in ORNs (Wu et al., 2003). A more detailed analysis of *Fst*^{-/-} OE revealed drastic depletion of cells at all ORN lineage stages (Fig. 1B,C): *Sox2*⁺ basal stem cells, *Ascl1*⁺ progenitors, *Neurog1*⁺ INPs and *Ncam*⁺ ORNs. Normally, *Sox2* is expressed in two distinct zones: in basal OE there are scattered *Sox2*⁺ neural stem cells (Kawauchi et al., 2005; Kawauchi et al., 2009) and the apical surface monolayer of OE Sus cells is also *Sox2*⁺ (Fig. 1B) (Guo et al., 2010). In *Fst* mutants, only the basal population of *Sox2*⁺ neural stem cells is depleted (Fig. 1B, see Fig. S1 in the supplementary material). This suggests that FST primarily affects neurogenesis, and not gliogenesis, in OE development, consistent with an inhibitory effect of FST on GDF11 function.

If FST acts solely by antagonizing GDF11, then *Fst*^{-/-}; *Gdf11*^{-/-} OE should display the *Gdf11*^{-/-} phenotype because absence of FST should have no effect when there is no GDF11 to antagonize. As shown in Fig. 1C, the numbers *Sox2*⁺, *Ascl1*⁺, *Neurog1*⁺ and *Ncam*⁺-expressing cells are indeed greater in *Fst*^{-/-}; *Gdf11*^{-/-} than in *Fst*^{-/-} OE; however, they are not increased to the level in *Gdf11*^{-/-} OE. Indeed, the numbers of ORN lineage cells in *Fst*^{-/-}; *Gdf11*^{-/-} OE are all significantly below the values observed in wild type. These data imply that the mechanism(s) by which FST promotes OE neurogenesis can only partially be accounted for by antagonism of GDF11.

This hypothesis is further supported by the significant depletion of basal *Sox2*⁺ and *Ascl1*⁺ cells that we observe in *Fst*^{-/-} OE (Fig. 1B,C). Previously, we showed that GDF11 action on OE neuronal progenitor cells is confined to INPs (Wu et al., 2003). Consistent with this, *Gdf11*^{-/-} OE displays no change in the numbers of *Ascl1*⁺ and basal *Sox2*⁺ cells (Fig. 1B,C). Moreover, exposure of OE cultures to GDF11 (at 10-fold higher concentration than that which completely blocks proliferation of INPs) has no effect on the development of ASCL1⁺ cells (see Fig. S2 in the supplementary material). Together, these observations suggest that a second TGFβ ligand – one that is antagonized by FST but is distinct from GDF11 – is likely to control the production of *Sox2*- and *Ascl1*-expressing stem and progenitor cells.

Activin βB is expressed by OE neuronal cells

The TGFβ ligands GDF11, GDF8, BMP7 and activins are bound with high affinity and neutralized by FST (Sidis et al., 2006; Schneyer et al., 2008). Of these, FST exhibits strongest binding to activins, GDF8 and GDF11. Since *Gdf8* is not expressed in OE (Wu et al., 2003), we focused on activins as potential candidates for the additional FST-sensitive factor(s) that regulate OE neurogenesis.

Activins are homo- or heterodimers of β-subunits, of which only *ActfβA* and *ActfβB* (also known as *Inhba* and *Inhbb*, respectively) are expressed in neural tissues (Fang et al., 1997; Chang et al., 2002). As shown in Fig. 1D, *ActfβA* is not expressed in OE (although it is transiently expressed in underlying mesenchyme from E12.5-14.5; data not shown). By contrast, *ActfβB*, like *Gdf11*, is expressed within OE proper. *Fst* expression is limited to the mesenchyme underlying OE prior to E14.5 (data not shown), but from E14.5 onward cells within the OE proper produce *Fst* as well (Fig. 1D) (Kawauchi et al., 2004).

Increase in stem and early progenitor cells in *ActfβB*^{-/-} OE

When we analyzed tissue from *ActfβB*^{-/-} mice, we found increases in both SOX2⁺ neural stem cells and ASCL1⁺ progenitors (Fig. 2). There were 22% more basal SOX2⁺ cells and 62% more ASCL1⁺

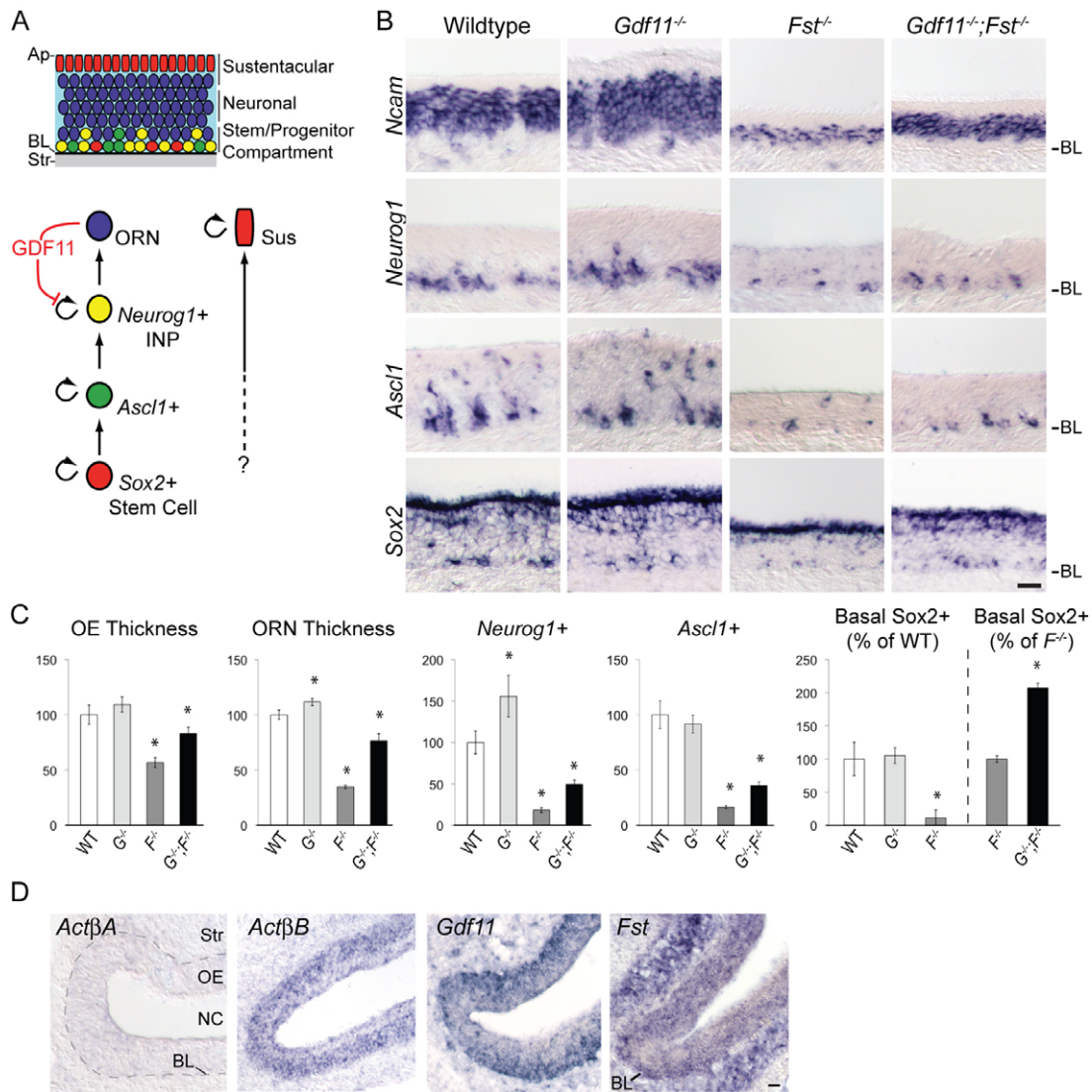


Fig. 1. Partial recovery of neurogenesis in *Gdf11*^{-/-};*Fst*^{-/-} mouse olfactory epithelium (OE). (A) Schematic of OE structure and lineage. The origin of sustentacular (Sus) cells is uncertain. (B) In situ hybridization (ISH) for the indicated markers. (C) OE thickness (μm), olfactory receptor neuron (ORN; *Ncam*⁺) layer thickness (μm), and *Neurog1*⁺, *Ascl1*⁺ and basal *Sox2*-expressing cells are plotted per mm OE and normalized as a percentage of wild type. Error bars indicate root mean square; *, *P* < 0.05 by Dunnett's test [DT (Glantz, 2005)]. (D) ISH with the indicated probes. BL, basal lamina; NC, nasal cavity; Str, stroma. Scale bars: 20 μm.

cells in *ActβB*^{-/-} OE than in controls. In addition, 30% more cells – predominantly basal in location – incorporated a short pulse of BrdU, indicating that the number of proliferating stem/progenitor cells is increased in *ActβB*^{-/-} OE. These findings are notable for two reasons. First, the increase in the number of SOX2- and ASCL1-expressing cells and in BrdU-incorporating basal cells demonstrates that ACTβB normally restrains the expansion of these populations. Second, whereas the magnitude of effects observed in *ActβB*^{-/-} OE is similar to that observed in *Gdf11*^{-/-} OE (22-62% increase, depending on cell type), the cell types affected are different [e.g. the levels of ASCL1⁺ cells are unchanged in *Gdf11*^{-/-} OE (Fig. 1B,C)].

ACTβB has an anti-neurogenic action distinct from that of GDF11

To determine whether ACTβB acts directly on ASCL1⁺ progenitors, we performed short-term OE explant cultures. In such cultures, ASCL1⁺ progenitors migrate away from explants (Fig. 3A, 'e'),

where they can be identified by immunostaining and quantified (Fig. 3A, white arrowheads). Treatment with activin B (ACTB homodimer of ACTβB subunits) for 8 hours leads to a dramatic reduction in ASCL1⁺ cells compared with controls, and addition of FST completely abrogates the effect of ACTB (Fig. 3B). FST alone has no effect on ASCL1⁺ progenitors, as expected (Wu et al., 2003).

Despite its pronounced effect on ASCL1⁺ progenitors, ACTB has no effect on the development of INPs (Fig. 3C) in a culture paradigm that assesses effects on INPs specifically (see Materials and methods). By contrast, GDF11 strongly inhibits INP development in these cultures, as expected (Wu et al., 2003). These results parallel what we observe in vivo: in *ActβB*^{-/-} OE, the number of *Neurog1*⁺ INPs is no different from wild-type OE (Fig. 3D), whereas in *Gdf11*^{-/-} OE this number is significantly increased (Fig. 1B,C).

Interestingly, we found that 2 hours of exposure to ACTB is sufficient for inhibition of ASCL1 expression in OE cultures (Fig. 3E), suggesting a direct action of ACTB on ASCL1⁺ cells,

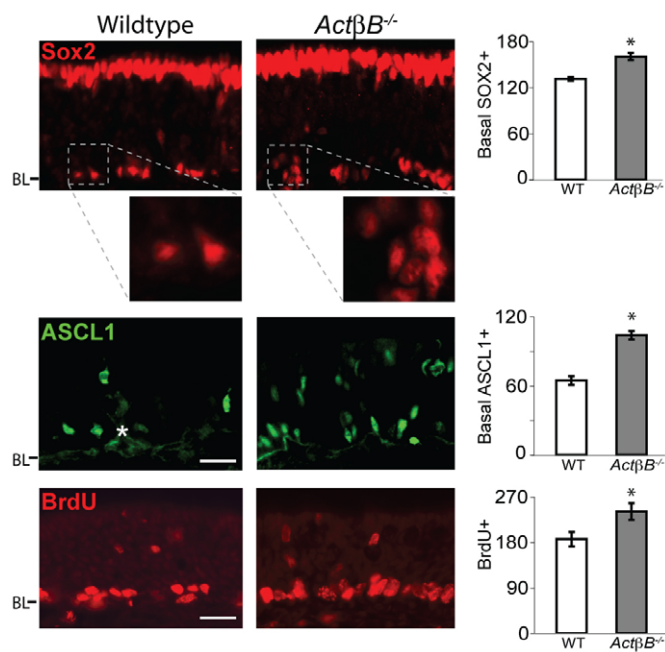


Fig. 2. Increase in SOX2⁺ and ASCL1⁺ cells in *Actfβ*^{-/-} OE. Bar charts show cells/mm OE (mean ± s.e.m.; *, $P < 0.05$ DT). White asterisk, Bowman's gland. BL, basal lamina. Scale bars: 20 μm.

reminiscent of that seen for BMP2 and BMP4: BMP treatment causes rapid, proteasome-mediated degradation of ASCL1 in OE cultures, resulting in arrest of ASCL1⁺ progenitor cell development and cessation of neurogenesis (Shou et al., 1999). Since the proteasome pathway has a known role in regulating cell differentiation (e.g. Reavie et al., 2010), we tested whether inhibition of this pathway prevents the effect of ACTB on ASCL1⁺ progenitors. As shown in Fig. 3E, the proteasome inhibitor MG132 (Rock et al., 1994) completely blocks the effect of ACTB on ASCL1⁺ cells. GDF11 had no effect on ASCL1⁺ cells in these cultures, nor did addition of MG132 alter this (Fig. 3E). Altogether, these findings indicate that ACTB and GDF11 exert distinct anti-neurogenic effects at different stages in the ORN lineage (Fig. 3F).

Differential expression of type I signaling receptors for ACTβB and GDF11

How might ACTβB and GDF11, which activate similar signaling pathways, exert such different effects? One possibility is that receptors specific for GDF11 and ACTβB are differentially expressed. Although activins and GDF11 can utilize the same type II ligand-binding receptors (Attisano et al., 1996; Oh et al., 2002), increasing evidence indicates that ACTβB and GDF11 signal preferentially through different type I receptors, with ALK4 (also known as ACVRIB and ACTRIB) being the preferred receptor for ACTβB, and ALK5 (TGFβR1) the preferred receptor for GDF11 (Attisano et al., 1996; Andersson et al., 2006; Tsuchida et al., 2008). We examined expression of *Alk4* and *Alk5* by ISH, and found that both are expressed in OE neuroepithelium (but not underlying stroma) from E12.5-17.5 (Fig. 4A). As development proceeds, *Alk4* expression remains widespread throughout the apical-basal extent of the OE, whereas *Alk5* expression becomes progressively restricted to basal OE, where stem and progenitor cells reside (Fig. 4B).

To gain further insight, we took advantage of *Ascl1*^{-/-} mice, in which OE neurogenesis is halted at the stage of *Ascl1*-expressing early progenitor cells (Guillemot et al., 1993; Murray et al., 2003; Wu et al., 2003). By E17.5, there are essentially no INPs or ORNs in *Ascl1*^{-/-} OE; instead, virtually all OE cells express markers of Sus cells and/or stem cells, including *Sox2*, *Otx2*, *Raldh3* (*Aldh1a3*) and *Steel* (*Kitl*) (Murray et al., 2003; Kawauchi et al., 2004; Beites et al., 2005). We found that expression of *Alk4* persists, but *Alk5* expression is absent, in *Ascl1*^{-/-} OE (Fig. 4C). This finding is consistent with the idea that *Alk4* expression reflects a bias toward glial (Sus) differentiation, whereas *Alk5* expression reflects a bias toward neuronal (ORN) differentiation (e.g. by INPs). The high-magnification images in Fig. 4D illustrate the fact that the patterns of expression of *Alk5*, *Gdf11* and *Neurog1* are highly similar, consistent with the finding that GDF11-mediated feedback acts primarily on INPs.

Insensitivity of neuron number to loss of ACTβB feedback

If the model of OE neurogenesis in Fig. 3F is correct, it might be expected that the excess of SOX2⁺ and ASCL1⁺ stem and early progenitor cells that accumulate in *Actfβ*^{-/-} OE would eventually translate into proportional increases in the numbers of INPs and ORNs (Gokoffski et al., 2009; Lander et al., 2009). Surprisingly, this is not the case: INP number and the thickness of the *Ncam*⁺ ORN layer (an indication of ORN number) are unchanged in *Actfβ*^{-/-} OE (Fig. 3D, see Fig. S3 in the supplementary material). Overall OE thickness (the distance from the basal lamina to the apical surface) is also unchanged [wild type, 67 ± 3 μm; *Actfβ*^{-/-}, 67 ± 7 μm (±s.e.m.)].

One obvious explanation is that GDF11, still present in *Actfβ*^{-/-} OE, limits INP divisions and ORN differentiation (Lander et al., 2009), thereby preventing an increase in INP and ORN numbers. We tested this idea by examining the OE of mice null for both *Actfβ* and *Gdf11* (Fig. 5). The absence of both *Actfβ* and *Gdf11* does not translate into overproduction of ORNs and INPs: numbers of *Neurog1*⁺ INPs and *Ncam*⁺ ORNs are the same in *Actfβ*^{-/-}; *Gdf11*^{-/-} OE and *Gdf11*^{-/-} OE, and the expansion of *Ascl1*⁺ and basal *Sox2*⁺ cells is at least as great as that seen in the absence of *Actfβ* alone (Fig. 5A, see Fig. S4 in the supplementary material). Therefore, negative feedback of GDF11 onto INP proliferation and/or differentiation cannot account for the selective increase in stem and early progenitor cells seen in *Actfβ*^{-/-} OE.

Interestingly, we did observe a striking increase in *Sox2* expression in the apical compartment of *Actfβ*^{-/-}; *Gdf11*^{-/-} OE (Fig. 5A, top panel, white arrowhead), suggesting that double mutants have increased numbers of Sus cells. To confirm this, we performed SOX2 IF in mice null for *Actfβ*, *Gdf11*, or both genes, and counted apical SOX2⁺ Sus cells. As shown in Fig. 5B-D, the number and spatial arrangement of these cells are not significantly different in *Actfβ* mutants compared with wild-type controls. By contrast, SOX2⁺ Sus cells are significantly increased in *Gdf11*^{-/-} OE, and even more so in *Actfβ*^{-/-}; *Gdf11*^{-/-} OE. These supernumerary SOX2⁺ Sus cells acquire immunoreactivity for SUS4 (Fig. 5C), a marker of differentiated Sus cells (Goldstein and Schwob, 1996; Huard et al., 1998). Together, these results demonstrate that the mature Sus cell population is expanded significantly in the absence of *Gdf11*, and even more dramatically when both *Gdf11* and *Actfβ* are absent. Moreover, although Sus cells are a self-renewing population (Weiler and Farbman, 1998), their expansion in *Gdf11*^{-/-} and *Actfβ*^{-/-}; *Gdf11*^{-/-} OE is not due to increased proliferation: BrdU labeling experiments showed that the

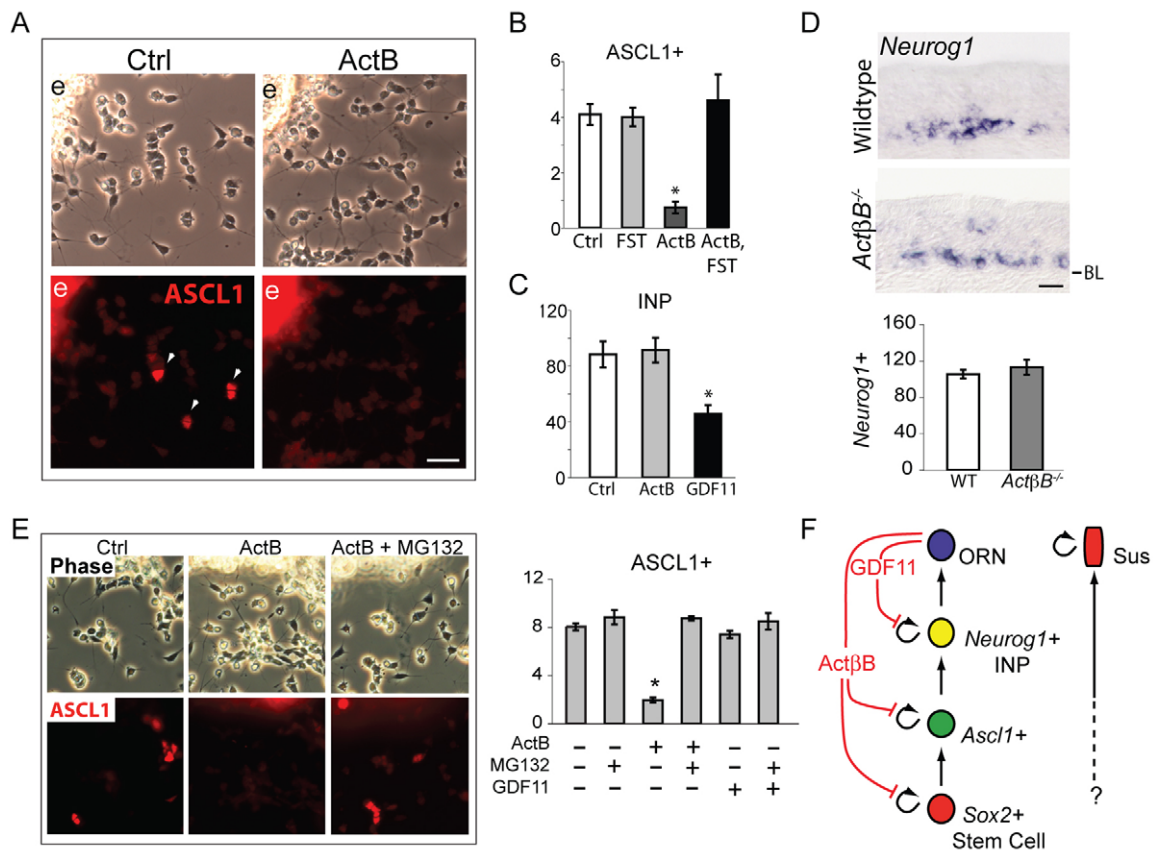


Fig. 3. ACTB limits development of ASCL1⁺ cells in vitro. (A,B) Mouse OE explants ('e') cultured for 8 hours in ACTB (10 ng/ml) and/or FST (200 ng/ml). White arrowheads indicate ASCL1⁺ cells. Bar chart shows percentage of cells that are ASCL1⁺ (mean ± s.e.m.; *, $P \leq 0.05$ DT). (C) *TgN1-2G^{+/+}* explants were cultured for a total of 30 hours, with ACTB (20 ng/ml) or GDF11 (20 ng/ml) added for the final 18 hours in vitro. GFP⁺ (*Neurog1*⁺) immediate neuronal precursors (INPs) were analyzed as described (Wu et al., 2003) and are plotted as mean (± s.e.m.) per 15,000 μm^2 explant. *, $P \leq 0.05$ by DT. (D) *Neurog1* ISH in *ActβB^{-/-}* and control OE. Bar chart shows mean ± s.e.m. (E) OE explants were cultured for 6 hours, then ACTB or GDF11 (20 ng/ml) was added for 2 hours. MG132 (10 μM) or control (0.1% DMSO in medium) was added 30 minutes prior to ACTB or GDF11 addition. Analysis as in A. (F) Schematic showing actions of ACTβB and GDF11 on the ORN lineage. BL, basal lamina. Scale bars: 20 μm .

proliferation index of Sus cells is the same in mutant and wild-type OE (Fig. 5E). These data make it unlikely that Sus cell self-renewal is regulated by GDF11 and/or ACTβB.

Altogether, these results suggest an alternative explanation for why additional early stage stem cells and progenitors in *ActβB^{-/-}* OE fail to produce supernumerary INPs and ORNs: these cells now preferentially give rise to Sus cells. There have been indications from previous work that Sus cells and ORNs might share a common progenitor in the OE (Huard et al., 1998; Murray et al., 2003; Chen et al., 2004; Kawauchi et al., 2004; Beites et al., 2005). The observation that Sus cells express SOX2, just as basal neuronal stem cells do, supports this idea (Kawauchi et al., 2004; Beites et al., 2005; Kawauchi et al., 2005; Guo et al., 2010). Altogether, our observations suggest that expansion of Sus cells in *ActβB^{-/-}; Gdf11^{-/-}* OE is due to increased production of Sus cells by SOX2⁺ basal stem cells.

Ascl1-expressing cells give rise directly to Sus cells

Alteration(s) in gliogenesis resulting in the diversion of excess SOX2⁺ stem cells into Sus cells provide a partial explanation for the insensitivity of neurogenesis to loss of feedback from ACTβB. However, it still does not explain why the excess in *Ascl1*⁺ cells in *ActβB^{-/-}; Gdf11^{-/-}* OE fails to translate into increased numbers of

INPs and ORNs. Although previous findings had suggested that ASCL1 marks cells specific to the ORN lineage (Gordon et al., 1995; Cau et al., 1997; Cau et al., 2002), we noticed that some ASCL1⁺ cells are present in or near the Sus cell layer in IF and ISH experiments (Fig. 2, Fig. 5A). We wondered whether this was an indication that ASCL1⁺ cells also give rise to Sus cells.

To address this, dual IF labeling for SOX2 and ASCL1 was performed. Fig. 6A shows that cells expressing both SOX2 and ASCL1 occur not only in the basal (stem/progenitor) compartment of the OE, as expected, but also in the apical (Sus) compartment. This, again, suggests that ASCL1⁺ cells might give rise directly to apical Sus cells. To test this idea directly, we used mice heterozygous for an allele in which the *Ascl1* coding sequence has been replaced with GFP [*Ascl1^{GFP/+}* mice (Leung et al., 2007)]. Because of the relatively long half-life of GFP (Okabe et al., 1997; Feng et al., 2000; LeSauter et al., 2003), fluorescence is detected not only in cells that are actively transcribing *Ascl1*, but also transiently in their progeny. Fig. 6B shows that the OE of these mice contains *Ascl1*-GFP⁺ cells (i.e. cells expressing GFP from the *Ascl1* locus) in the apical Sus cell layer. These apical *Ascl1*-GFP⁺ cells resemble Sus cells morphologically and also express the differentiated Sus cell marker SUS4 (Fig. 6B, bottom panel) (Goldstein and Schwob, 1996; Huard et al., 1998).

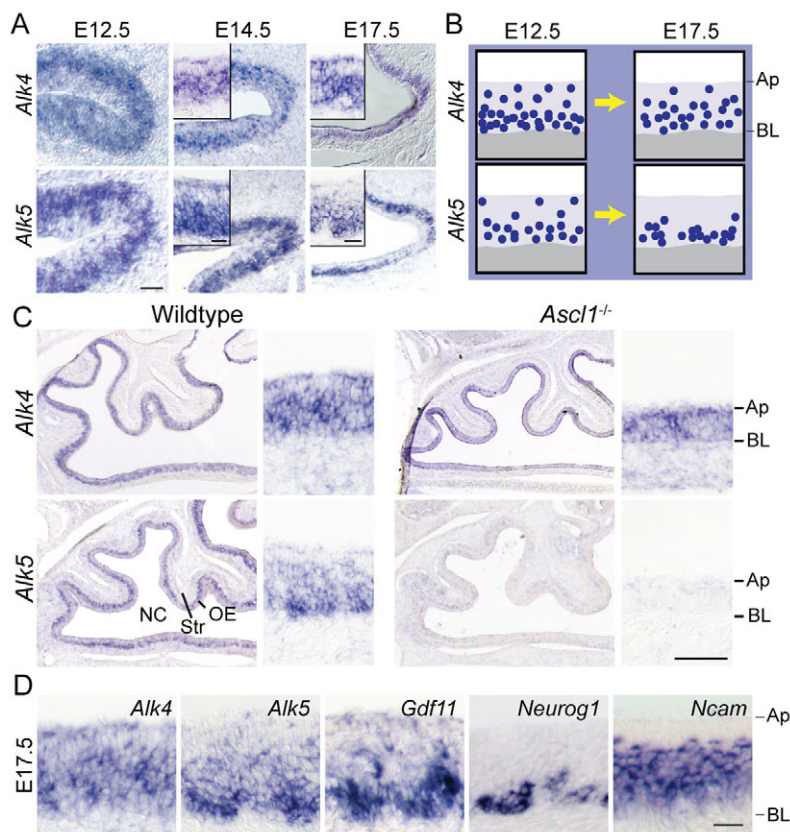


Fig. 4. Expression of *Alk5*, but not *Alk4*, requires

***Ascl1* function. (A)** ISH in wild-type mouse OE.

(B) Schematic of developmental changes in *Alk4* and *Alk5* expression. Blue circles, represent *Alk4*- or *Alk5*-expressing cells. **(C)** ISH in *Ascl1*^{-/-} and wild-type OE. **(D)** Wild-type OE hybridized with the indicated probes. Ap, apical surface; BL, basal lamina; NC, nasal cavity; Str, stroma. Scale bars: 50 μm in C, A E12.5; 20 μm in D, A E14.5 and E17.5.

To confirm that *Ascl1*-expressing cells give rise directly to Sus cells, we performed inducible lineage-tracing experiments in which *Ascl1*^{CreERT2/+} mice were crossed with R26R-stop-YFP reporter mice. Pregnant dams were injected with tamoxifen (Tmx), causing *Ascl1*⁺ cells and their descendants to become marked by YFP (see Materials and methods). When the OE of pups was examined at P0, YFP⁺ cells that also expressed SUS4 were readily observed (Fig. 6C). Altogether, these data indicate that some Sus cells derive directly from cells that express *Ascl1* and, thus, that expression of *Ascl1* does not irreversibly commit OE progenitor cells to a neuronal fate.

If becoming Sus (glial) is a normal fate for an *Ascl1*⁺ progenitor, then one might expect to observe the migration, during development, of *Ascl1*⁺ Sus cells from a reservoir of basal *Ascl1*⁺ progenitors. We performed BrdU pulse-chase experiments using *Ascl1*^{GFP/+} mice. As shown in Fig. 6D-F, 2 hours following a pulse of BrdU, virtually all GFP⁺ BrdU⁺ cells (i.e. *Ascl1*-expressing cells that take up BrdU) are located in basal OE. Twenty-four hours following a BrdU pulse, however, many double-labeled cells have left the basal stem/progenitor compartment and are now in the apical Sus cell compartment. These phenomena could only arise if (1) most or all apical GFP⁺ cells that were labeled with BrdU during the pulse underwent a round of replication during the 24-hour chase and remained in the Sus compartment, or (2) basal *Ascl1*-GFP⁺ progenitor cells, which incorporated BrdU while present in the basal compartment, subsequently migrated to the apical Sus compartment. Two observations indicate that the second alternative is correct. First, any change in the BrdU labeling index of Sus cells over the course of the chase is minimal, demonstrating that few Sus cells are self-replicating at this age [pulse-fix, 100±2 apical BrdU⁺ cells/mm OE; pulse-chase, 114±11 apical BrdU⁺

cells/mm OE (±s.d.)]. Second, during the same period in which the number of apical GFP⁺ BrdU⁺ cells rises (Fig. 6E), the number of basal GFP⁺ BrdU⁺ progenitor cells decreases by half (Fig. 6F). In order for these shifts to occur simultaneously, there must be an efflux of GFP⁺ BrdU⁺ cells from the basal stem/progenitor compartment into the apical compartment, indicating that basal *Ascl1*-expressing progenitors migrate to apical OE, where they differentiate into Sus cells.

These observations together argue in favor of a revision of the view that *Ascl1* expression marks a neuronally committed stage on the path from *Sox2*⁺ stem cell to ORN. Instead, they raise the possibility that there is a single, bipotential stem/progenitor cell that is capable of expressing *Sox2*, *Ascl1* or both (Fig. 6G). Cues that bias the amount of time spent in each expression state, or the level of each transcription factor, would then influence the proportion of progeny that differentiate along neuronal versus glial lines. This view is further supported by the results described below.

***ActβB* and *Gdf11* modulate allocation of OE stem cells to different fates**

Consideration of the model outlined in Fig. 6G prompted us to quantitatively analyze SOX2 and ASCL1 single- and double-immunopositive cells in *Gdf11*^{-/-}, *ActβB*^{-/-} and double-mutant OE, separately scoring cells that are in the basal stem/progenitor compartment versus the apical Sus compartment. Fig. 7A-C presents the results using stacked bar charts, in which numbers of single- and double-immunopositive cells of each type (per mm OE) are reflected in bar heights and the proportions of each type are given by percentage (for absolute cell counts, see Table S1 in the supplementary material).

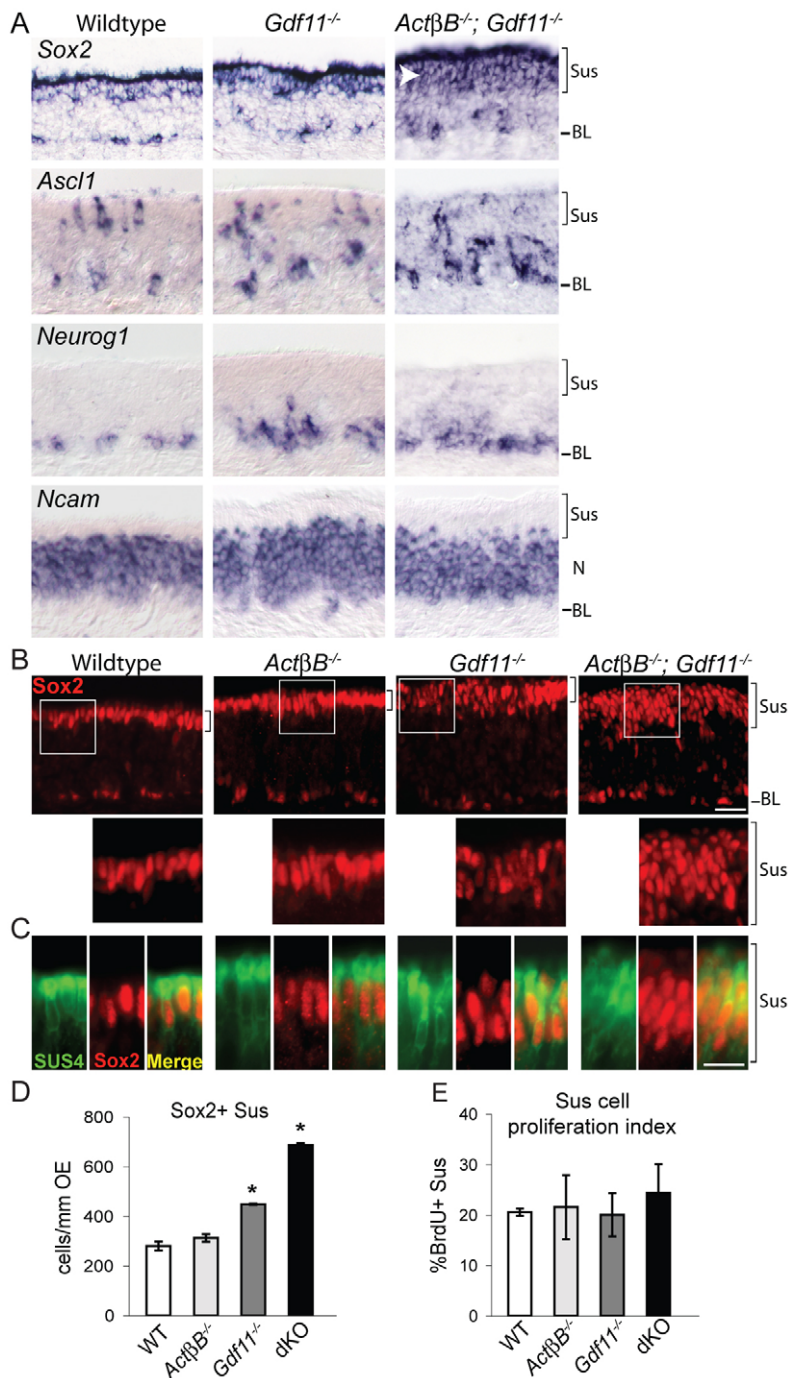


Fig. 5. Increased Sus cells in *Gdf11*^{-/-} and *ActβB*^{-/-};*Gdf11*^{-/-} OE. (A) ISH with the indicated probes indicates increased Sox2 expression and a thicker Sus layer (white arrowhead) in *ActβB*^{-/-};*Gdf11*^{-/-} OE. (B) Magnified images show apical SOX2⁺ cells outlined by white boxes. (C) Most apical SOX2⁺ cells are also SUS4⁺ [83±2% (±s.e.m.)]. (D) Mean number of SOX2⁺ Sus cells/mm OE (±s.e.m.; *, *P*≤0.05 DT). (E) SOX2⁺ Sus cells and the percentage of these that are BrdU⁺ ('proliferation index') plotted as mean ± s.e.m. dKO, *ActβB*^{-/-};*Gdf11*^{-/-}. BL, basal lamina; N, neuronal layer; Sus, sustentacular layer. Scale bars: 20 μm in A,B; 10 μm in C.

In *ActβB*^{-/-} OE, where we had earlier noted simply that basal SOX2⁺ and ASCL1⁺ cells are both increased (Fig. 2), it is now possible to see that this effect is due solely to an increase in ASCL1⁺ and in SOX2⁺; ASCL1⁺ cells. Cells that express SOX2 alone are not significantly increased in number compared with wild type. These effects are observed in both the basal stem/progenitor compartment (Fig. 7B) and the apical Sus compartment (Fig. 7C). In *Gdf11*^{-/-} OE, we previously noted that the numbers of basal SOX2⁺ and ASCL1⁺ cells are not significantly different from wild type (Fig. 1C) (Wu et al., 2003), which was confirmed here (Fig. 7B). However, if we combine the data from the apical and basal compartments (Fig. 7A), we see a large increase in the total number of SOX2 single-positive cells, a possible small increase in SOX2⁺ ASCL1⁺ cells, and no change in ASCL1 single-positive cells. In

ActβB^{-/-};*Gdf11*^{-/-} OE, where we had noted an even larger expansion of basal SOX2⁺ cells than occurs in the absence of *ActβB* alone (compare Fig. 5A with Fig. 2), we could now see that the additional expansion is due to an increase in the number of cells that express SOX2 alone, as opposed to both SOX2 and ASCL1 (Fig. 7B, see Table S1 in the supplementary material). This is precisely the population that is not expanded in the *ActβB* mutant. The same trends are observed in the apical compartment, where only SOX2 single-positive cells are significantly increased in number compared with what is seen in *ActβB*^{-/-} OE (see Table S1 in the supplementary material).

A coherent picture emerges from these data: loss of *ActβB* favors the selective expansion of cells with a 'neuronal bias' (i.e. those expressing ASCL1 alone or together with SOX2), whereas loss of

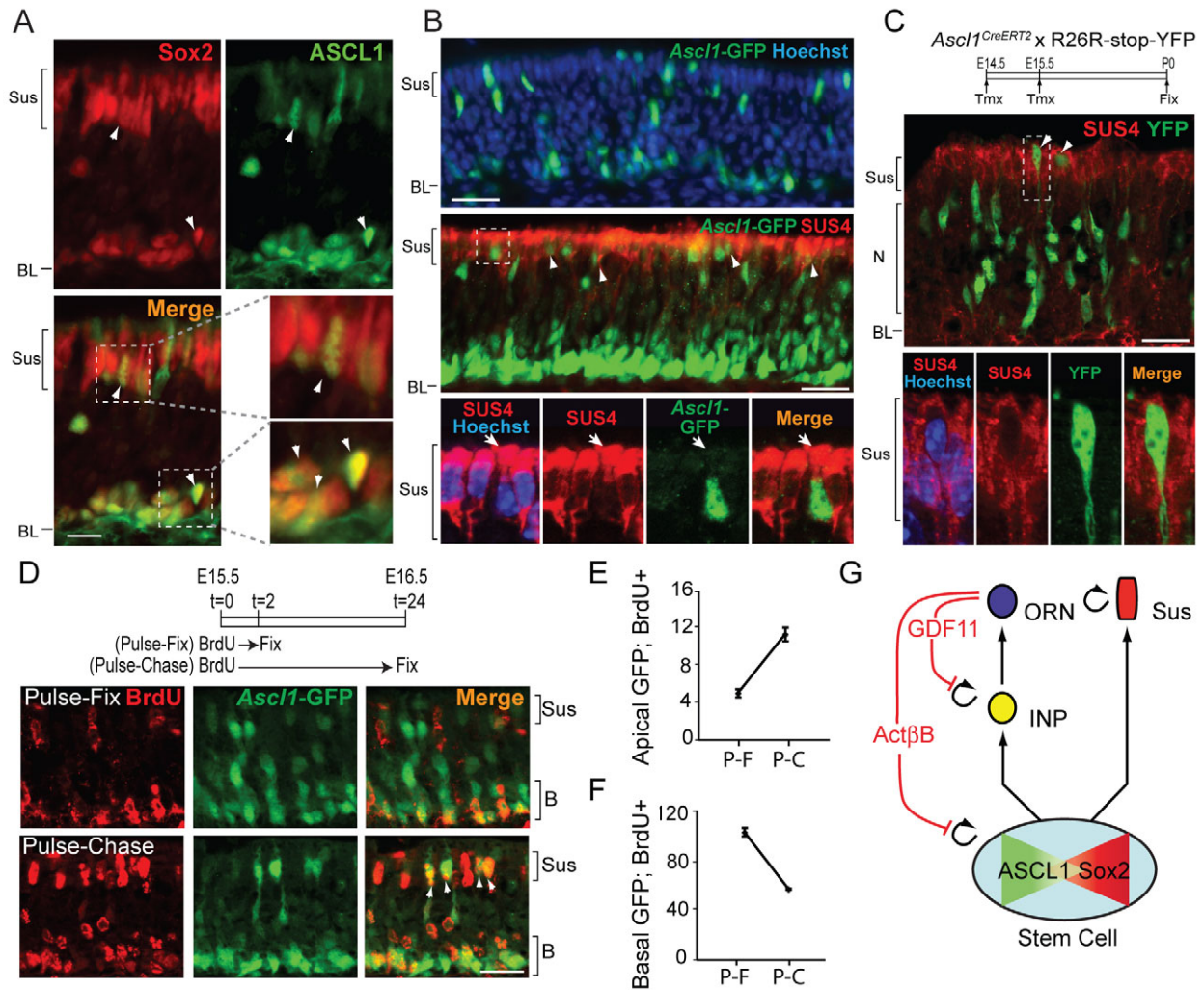


Fig. 6. *Ascl1*-expressing cells give rise directly to Sus cells. (A) Immunofluorescence (IF) for SOX2 and ASCL1 in wild-type OE. White arrowheads indicate SOX2⁺; ASCL1⁺ cells. (B) In *Ascl1*^{GFP/+} OE, apical GFP⁺ cells are SUS4⁺ (arrowheads). The bottom row shows a z-stack image (2 μ m spacing) of the boxed cell. Arrows indicate a SUS4⁺; GFP⁺ cell. (C) Pregnant dams were injected with tamoxifen (Tmx; see Materials and methods) and SUS4 IF performed on P0 OE. The bottom row shows a z-stack image (0.5 μ m spacing) of the boxed cell. (D) BrdU was administered to pregnant dams (*Ascl1*^{GFP/+}) and GFP⁺ and BrdU⁺ cells analyzed in Sus and basal ('B') compartments at the times indicated. (E,F) Quantification of experiments in D, plotted as mean GFP⁺ BrdU⁺ cells/mm OE (\pm s.d.). P-F, pulse-fix; P-C, pulse-chase. (G) Schematic showing feedback and dynamic expression of *Sox2* and/or *Ascl1* by a stem cell. BL, basal lamina; N, neuronal layer; Sus, sustentacular layer. Scale bars: 20 μ m.

Gdf11 favors the selective expansion of cells with a 'glial bias' (i.e. SOX2 single-positive cells). In the latter case, most of the additional SOX2⁺ cells move to the apical compartment, where they produce supernumerary Sus cells [this explains why there is no increase in early stage neuronal progenitors (ASCL1 single-positive cells) in the *Gdf11* single mutant].

These results suggest that ACT β B promotes stem/progenitor cell commitment to the glial (Sus) fate, whereas GDF11 inhibits commitment to that fate. To examine this directly, we tested the effects of ACTB and GDF11 on the development of Sus cells in vitro, in OE explant cultures grown under conditions optimized for Sus cell development (see Materials and methods). In such cultures, Sus cells can be visualized as coherent epithelial sheets in which virtually all cells express cytokeratin 18 (CYT18; also known as keratin 18); Fig. 7D shows CYT18 staining of Sus cells in the OE in vivo (see Pixley, 1992; Jang et al., 2008). To determine the effects of ACTB and GDF11 on Sus cell development in vitro, cultures were grown for 48 hours in the presence or absence of these factors.

During the final 24 hours, BrdU was added, following which cultures were fixed and processed for BrdU and CYT18 immunoreactivity (Fig. 7E, diagram).

As predicted from our findings in vivo, ACTB had a positive effect, whereas GDF11 had a negative effect, on Sus cell development in vitro (Fig. 7E). When we quantified double-label IF experiments, we found that the percentage of definitive Sus cells (CYT18⁺) derived from BrdU-labeled cells was 57% greater in ACTB-treated cultures than in controls. Conversely, cultures treated with GDF11 contained 44% fewer double-labeled cells than controls (Fig. 7E).

Importantly, when experiments were performed in which Sus cell cultures were exposed for 16 hours to GDF11 or ACTB and then pulsed for 2 hours with BrdU, no substantial effect on the proliferation of existing Sus cells was observed with either factor (data not shown). This finding is reminiscent of what is observed in vivo in *Gdf11*^{-/-}, *Act β B*^{-/-} and *Gdf11*^{-/-}; *Act β B*^{-/-} OE, where no significant difference in the BrdU pulse-labeling index of Sus cells is seen between any of these mutants and wild-type littermates (Fig.

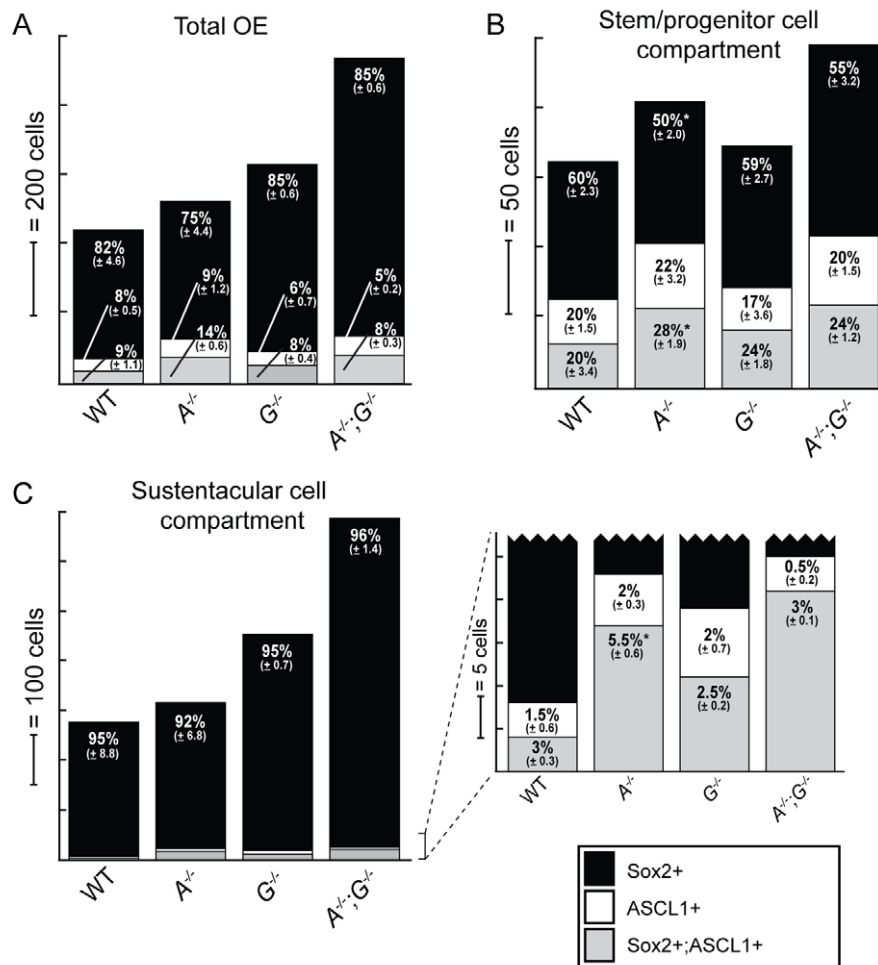
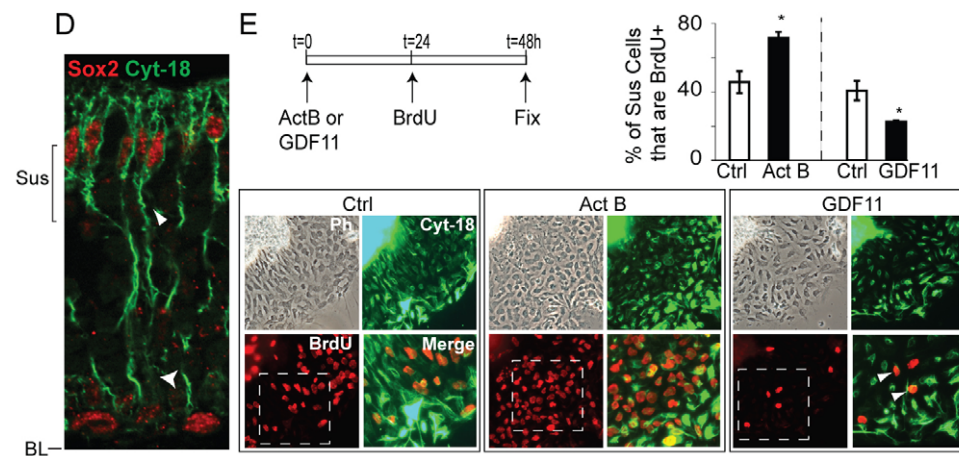


Fig. 7. *ActβB* and *GDF11* modulate stem cell fates. (A-C) Cells in three marker categories (SOX2⁺, black; ASCL1⁺, white; SOX2⁺; ASCL1⁺, gray) were quantified for (A) total OE, (B) basal stem/progenitor cell compartment and (C) apical sustentacular cell compartment. The height of each shaded segment in a bar indicates the total number of cells/mm OE for that category. The percentage of cells in a given compartment [± error (root mean square)] for each marker category is indicated. *, *P* ≤ 0.05 (DT) compared with wild type. *A*, *ActβB*; *G*, *Gdf11*. (D) z-stack image (0.3 μm spacing) of SOX2 and cytokeratin 18 IF. White arrowheads indicate a Sus cell that is SOX2⁺; CYT18⁺. (E) Sus cultures were treated with ACTB (20 ng/ml) or GDF11 (20 ng/ml). BrdU (1:10,000) was added for the final 24 hours in vitro. Bar charts show mean ± s.e.m. (*, *P* ≤ 0.05, Student's *t*-test). White arrowheads in the magnified image point to BrdU⁺ CYT18⁺ cells. Ph, phase contrast image; BL, basal lamina; Sus, sustentacular layer.



5E). Altogether, our findings in vivo and in culture indicate that ACTβB and GDF11 exert opposing effects on Sus cell development, with ACTβB promoting, and GDF11 opposing, the production of Sus cells.

DISCUSSION
GDF11 and ACTβB have distinct, cell stage-specific feedback actions on neurogenesis and gliogenesis

Here we show that two TGFβ superfamily signaling molecules, GDF11 and ACTβB, play crucial and distinct roles in feedback regulation of stem and progenitor cell proliferation and fate in the

OE (Fig. 8). In the neurogenic pathway, GDF11 inhibits proliferation of INPs, the progenitor cells that occupy the penultimate place in the ORN lineage (Wu et al., 2003; Lander et al., 2009), whereas ACTβB inhibits expansion of the cells that give rise to INPs. These latter progenitors, which express *Sox2* and/or *Ascl1*, had previously been treated as two distinct populations – the *Sox2*⁺ stem and *Ascl1*⁺ transit-amplifying cell stages (Calof et al., 2002; Kawauchi et al., 2004; Beites et al., 2005; Kawauchi et al., 2005). However, the present study shows that basal compartment cells that express *Sox2* and/or *Ascl1* contribute to both the ORN and Sus cell populations, arguing that the fluctuating expression of

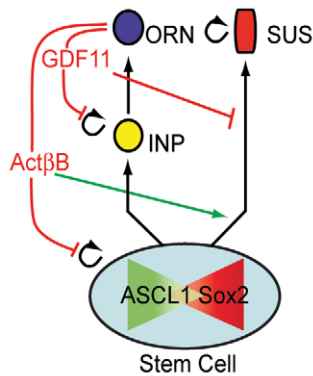


Fig. 8. Schematic of OE lineages showing distinct roles for GDF11 and ACT β B feedback. In the ORN pathway, GDF11 feedback limits INP proliferation, whereas ACT β B feedback inhibits proliferation of stem cells that give rise to INPs. In the Sus pathway, GDF11 antagonizes, whereas ACT β B promotes, stem cell development into Sus cells.

these genes denotes a single population of bipotent stem/progenitor cells in a dynamic state. If these cells migrate apically and *Sox2* expression is maintained then they commit to a glial (Sus) fate (Figs 5-7). An interplay between SOX2 and bHLH transcription factors (such as ASCL1) in the establishment of neuronal versus glial identity has been seen in other instances (reviewed by Pevny and Placzek, 2005; Wegner and Stolt, 2005; Kondoh and Kamachi, 2010), but the results reported here show for the first time that the outcomes of that interplay are controlled by feedback provided by TGF β superfamily signaling molecules.

Altogether, our view of a dynamic stem/progenitor cell population in the OE is consistent with recent data in a variety of stem cell systems suggesting that the transition between stem and transit-amplifying cells is neither sharp nor irreversible (Xi and Xie, 2005; Zipori, 2005; Clayton et al., 2007; Frye et al., 2007; Jones et al., 2007), as well as with evidence that bipotential progenitor cells often co-express genes that specify mutually exclusive cell fates (a phenomenon sometimes called transcriptional priming) (Laslo et al., 2006; Glauche et al., 2007). An indication of these phenomena might in fact have been seen in experiments in which the OE of adult rodents was subjected to extensive damage: in some such cases, cells that do not normally participate in the ORN lineage, such as squamous 'horizontal basal epithelial cells', were able to contribute to the progenitor cell pool, and at least some of their progeny became ORNs (Huard et al., 1998; Jang et al., 2003; Leung et al., 2007; Iwai et al., 2008). Such findings argue that the stem/progenitor cell population of the OE is not fixed, and that production of neurons remains sensitive to environmental cues long after development has ceased.

Analysis of the production of Sus cells in *Gdf11*^{-/-}, *Act β B*^{-/-} and double-mutant mice, together with results from Sus cell cultures, indicate that ACT β B biases the OE bipotent stem/progenitor cell toward a glial fate, whereas GDF11 biases it toward the production of neurons (Fig. 5, Fig. 7). Although there were some indications from prior studies that OE stem cells might share properties with Sus cell progenitors (Murray et al., 2003), we were surprised to find that ASCL1⁺ cells can give rise to Sus cells, especially because previous reports from us and others indicated that *Ascl1* expression marks cells committed to the neuronal lineage in the OE (Gordon et al., 1995; Cau et al., 1997; Shou et al., 1999; Shou et al., 2000; Tomita et al., 2000; Cau et al., 2002). However, our finding that *Ascl1*-expressing cells can be progenitors for both ORNs and Sus cells brings the OE into line with the findings from recent studies of the

developing central nervous system, where it has been shown that *Ascl1* is expressed by subsets of progenitor cells that can give rise to glia, as well as neurons, in regions as diverse as the subventricular zone, spinal cord and corpus callosum (Parras et al., 2004; Battiste et al., 2007; Kim et al., 2007; Kim et al., 2008).

Importantly, our findings with *Act β B*^{-/-}, *Gdf11*^{-/-} and *Act β B*^{-/-}; *Gdf11*^{-/-} mutant mice demonstrate that it is the signaling environment that controls the balance of glial versus neuronal commitment by stem/progenitor cells in the OE. In addition, our observation that GDF11 and ACTB influence stem and progenitor cell fate, as well as proliferation, reconciles the actions of these factors in the OE with those that we have identified in other parts of the nervous system, such as the retina, where loss of GDF11 leads to alterations in cell fate without detectable changes in proliferation (Kim et al., 2005).

Direct versus indirect actions of ACT β B and GDF11

Because both proliferation and differentiation within the OE lineage are influenced by feedback (Lander et al., 2009), distinguishing direct from indirect phenotypic effects can be challenging. With respect to ACT β B, we can be reasonably confident that its influence on fate choice by bipotential stem/progenitor cells is direct. Supporting data include: (1) in vitro, ACTB acts directly on ASCL1⁺ cells to block their development (Fig. 3E), whereas it enhances development of Sus cells (Fig. 7E); (2) loss of *Act β B* function in vivo leads to an increase in the proportion and number of basal progenitor cells that express ASCL1 (with or without concomitant expression of SOX2; Fig. 2, Fig. 7B, see Table S1 in the supplementary material); (3) ACTB has no observable effects on INPs, in vitro (Fig. 3C) or in vivo (Fig. 3D); and (4) *Act β B* has no observable effect on the proliferation index of differentiated Sus cells (Fig. 5E).

With respect to GDF11, previous work showed that it acts directly on INPs both to decrease their rate of cell division and increase their probability of differentiating into ORNs (Wu et al., 2003; Lander et al., 2009). Here, we have uncovered an additional role for GDF11 in the Sus lineage: GDF11 inhibits the development of Sus cells in vitro (Fig. 7C,E) and loss of *Gdf11* function leads to an increase in Sus cell number in vivo (Fig. 5). If GDF11, like ACT β B, acts directly on stem/progenitor cells, then it will be necessary to figure out how two molecules that ostensibly utilize the same SMAD2/3 signaling pathway (Oh et al., 2002; Feng and Derynck, 2005) exert opposite effects on the same cell type, one imposing a bias toward glial fate and the other a bias toward neuronal fate. The alternative is that some or all of the effects of GDF11 on stem/progenitor cells are indirect, mediated through changes brought about by GDF11 in the numbers and proportions of INPs and ORNs. For example, if INPs produce a signal that acts on stem/progenitor cells to bias their progeny toward Sus fates, this could explain why Sus cell numbers rise in *Gdf11* mutants (i.e. the effect is an indirect consequence of the overproduction of INPs in these animals). Although we have no direct evidence for such a mechanism, this hypothesis brings to mind the known actions of Notch signaling in other systems. Indeed, Notch signals provided by neuronal cells are often used to drive progenitors toward non-neuronal fates – a feedback mechanism that ensures the production of neuronal and non-neuronal cells in balanced proportions (Gaiano and Fishell, 2002; Kageyama et al., 2005; Karcavich, 2005). As multiple Notch ligands and receptors are expressed in the OE (Lindsell et al., 1996; Cau et al., 2000; Doi et al., 2004; Manglapus et al., 2004; Carson et al., 2006; Orita et al., 2006; Schwarting et al., 2007), this idea merits future investigation. Interestingly, *Notch2* has recently been shown to be important for the maintenance of Sus cells in adult OE (Rodriguez et al., 2008).

Implications for the control of lineage progression and regeneration

The experiments presented here underscore the importance of feedback control in developing neural tissues. In most of the nervous system, where the capacity for neurogenesis declines precipitously over the course of development (Kauffman, 1968; Caviness et al., 1995; Brazel and Rao, 2004; Galvan and Jin, 2007; Gould, 2007), investigations with the goal of identifying conditions that promote regeneration in the adult nervous system have focused on finding factors that stimulate stem cell self-renewal and expansion (Doetsch et al., 2002; Lenington et al., 2003; Zheng et al., 2004). The work presented here, together with a growing body of literature in other tissues (Lee and McPherron, 2001; Gamer et al., 2003; Falk et al., 2008; Manceau et al., 2008), suggest that the management of feedback signals that inhibit self-renewal and cell-cycle progression and that bias cell fate decisions might be a much more important strategy in understanding how to create conditions permissive for neural regeneration and repair.

In addition, the findings we present indicate that there is a close connection between the signals involved in the control of tissue size and those that regulate the proportions of cells of different types within a given tissue. This is likely to be a key concept in understanding stem cell-based lineages in general: increasing evidence that feedback signals and cell-cell interactions control the diversity of cell types produced by stem cell lineages can be found in tissue and organ systems as diverse as *Drosophila* gut and central nervous system (Ohlstein and Spradling, 2007; Baumgardt et al., 2009), nematode genitalia (e.g. Yu et al., 2009) and vertebrate spinal cord and retina (Reh, 1987; Eisen, 1992; Kim et al., 2005). Indeed, one of the feedback regulators analyzed in the present study, GDF11, also plays a major role in determining the proportions of different neural cell types produced by the mammalian retina (Kim et al., 2005).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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