DYNAMICS AND REGULATION OF OLFACTORY PROGENITORS DURING EPITHELIAL DEVELOPMENT AND MAINTENANCE

by

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Abstract

In mammals, the olfactory epithelium (OE) is one of the few known sites where neurons are continually made throughout the lifetime of the animal. An in-depth understanding of the mechanisms that facilitate this process could lead to important breakthroughs in the treatment of neurological and psychiatric diseases. At present, neither the identity of the stem cell responsible for this neurogenic capacity nor the mechanisms that lead to their activation is completely understood. In this thesis, I employ novel strategies to conditionally interfere with TGFβ signaling, a known modulator of neurogenesis during development, allowing this pathway to be inhibited in the adult OE for the first time. We show that expressing high levels of the TGFβ inhibitor follistatin does not appear to alter neurogenesis in the adult OE. I concurrently utilized a genetic fate-mapping strategy to examine the dynamics of Mash1-expressing cells. We found that labeling of Mash1-expressing cells postnatally marked a population of transient amplifying cells that produces predominantly neurons. Unilateral lesioning of postnatally-labeled mice leads to the extinction of labeled cells on the lesioned side. In contrast, when labeled in utero, Mash1-expressing cells give rise to a variety of cell types that resemble sustentacular and horizontal basal cells. Together, these experiments support the hypothesis that the mechanisms involved in regulating development of the OE may be distinct from those that maintain it throughout life.

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Chapter I: Introduction to Olfactory Neurogenesis
In medicine, our knowledge of disease and its treatment has been greatly enhanced by our understanding of human biology at the cellular and molecular level. Rational and evidence-based therapies are developed as our knowledge of these processes are discovered. The complex nature of the brain has hampered the mechanistic understanding of neurological and psychiatric disease. Model systems have proven useful in unraveling the underlying cellular and molecular processes leading to normal development and disease. The mammalian olfactory epithelium (OE) is an ideal model system to understand the basic process of cell fate determination and neuroregeneration. It has a simple architecture, with only two mature cell types, a glial and neuronal cell type. Additionally, these olfactory sensory neurons (OSNs) make direct connections to the brain, are capable of regeneration, and are readily accessible for sampling. Leading hypotheses regarding the genesis of psychiatric diseases such as schizophrenia postulate a defect in neurodevelopmental programs as the primary dysfunction. Given the continual regeneration of the OE, it has been targeted as a tissue that can provide a snapshot into neurodevelopment in adults. The observation that CNS diseases, such as Alzheimer’s Disease, can also cause OSN pathology (Talamo, et al. 1989) strengthens the hope that understanding how they regenerate can lead to novel therapeutic modalities for brain diseases. However, it is unclear whether the mechanisms that underlie regeneration are the same as those utilized during development. Some advances have been made into understanding the signaling pathways that influence neurogenesis in the developing OE and the developmental pathways taken by progenitor cells. This work utilized novel techniques to study these same pathways in the adult to determine whether the same mechanisms are utilized.
Organization of the mammalian olfactory system

Olfaction is an important, ancient sense. Chemical detection allows organisms to navigate their environment, find food, avoid danger, and select mates. This critical ability is preserved across the spectrum of organisms, underlying the simple chemotaxis of bacteria (Wadhams and Armitage 2004). In mammals, the detection of volatile chemicals takes place in the olfactory epithelium (OE) (Figure 1.1a), a pseudostratified neuroepithelium found within the dorsal nasal cavity. In mice, this epithelium is highly turbinated, providing an extensive surface area for chemical detection. Odorants from the environment bind to olfactory receptors (ORs) located on specialized bipolar-shaped olfactory sensory neurons (OSNs). Adult mice have hundreds of millions of OSNs, each of which expresses only one of the ~1300 members of the OR superfamily (Reed, et al. 1992). While the process of OR choice is still incompletely understood, it is clear that only a single allele of the gene is activated (Chess, et al. 1994), and the activation of a functional OR is necessary and sufficient to inhibit the further activation of OR genes (Lewcock and Reed 2004).

By the close of the 19th century, His and Ramon y Cajal had independently demonstrated that OSN axons project directly into the forebrain (Crews and Hunter 1994). Binding of odorants to receptors leads to propagation of action potentials down these axons to the olfactory bulb (OB), where they synapse on second order neurons (mitral and tufted cells) and interneurons (periglomerular cells) (Shepherd 1972) (Figure 1.1b). Processing of odorant stimuli in the OB is aided by the topographic mapping inherent to the glomerular arrangement of synapses. Each glomerulus receives inputs
from OSNs expressing a single type of receptor. (Mombaerts, et al. 1996). The overall arrangement of OR expression in the epithelium derives in part from the zonal restriction of receptor expression. The OE is divided roughly into 4 zones in the dorsal-ventral plane, and a particular receptor is expressed in only 1 of the 4 zones (though expression within that zone is apparently random) (Mori, et al. 2000) (Figure 1.2). Signals are processed and refined in the bulb before being relayed to areas of cortex, which include the anterior olfactory nucleus, piriform cortex, anterior cortical nucleus of the amygdala, periamygdaloid cortex, and the entorhinal cortex (Kosaka and Kosaka 2005). The heavy connections to limbic areas likely underlie the strong emotions conveyed by olfaction and its capacity for generating powerful memories.

**Cellular and Molecular Functioning of OE**

The mature OE is a pseudostratified epithelium composed primarily of a glial-like cell, a mature neuronal cell, basal cells, and Bowman’s glands (Figure 1.3a). The sustentacular (sus) cells are a glial cell type that occupy the apical-most layer of the epithelium and send foot processes to the basement membrane (Klein and Graziadei 1983). The microvillar processes on their apical surfaces distinguishes them morphologically. They also express specific sets of genes, including cytokeratins (Pixley 1992), the transcription factor sox2 (Guo, et al. 2010), as well as cytochrome P450 proteins such as cyp2g1, presumably to help them serve in a neuroprotective role by neutralizing toxic substances (Gu, et al. 1999). Sus cells are capable of self-renewal (Mackay-Sim and Kittel 1991), but it is unclear whether this capacity is sufficient for them to maintain their abundance during normal cellular turnover.
The other mature cell type is the OSN, identifiable by its expression of neuronal markers including NCAM, as well as the OSN-specific proteins, olfactory marker protein (OMP) and the proteins involved in odorant-induced signal transduction. The cell bodies of these OSNs occupy the neuronal zone of the OE. These neurons send a single dendrite apically, with an elaborate array of cilia present above the sus cell layer, where ORs are found and odorant binding takes place, just a few microns below the surface of the air-mucus interface (Menco 1980). Odorant binding leads to signal transduction mediated by an olfactory-specific G protein cascade, which leads to the firing of action potentials in the odorant-stimulated OSN (Reed, et al. 1992).

In the basal zone of the OE, two distinct basal cell populations exist. Lining the basal lamina is a keratin-expressing (Suzuki and Takeda 1991), morphologically flat cell type known as horizontal basal cells (HBCs). This rarely-dividing cell type is capable of proliferating and differentiating into all OE cell types when isolated in vitro (Carter, et al. 2004) or after severe chemical lesion of OE (Leung, et al. 2007). HBCs express the p53 tumor suppressor-related protein p63, the expression of which appears to be necessary for HBC formation and prevents differentiation of HBCs (Fletcher, et al. 2011; Packard, et al. 2011). The p63 protein expression is downregulated when HBCs are activated to heal OE wounds after chemical lesion (Packard, et al. 2011). Found just apical to the HBCs are the globose basal cells (GBCs), a heterogeneous population of keratin-negative cells that is mitotically active, and capable of producing all mature cell types in the OE (Caggiano, et al. 1994; Schwob, et al. 1994). A subset of these cells express Sox2, and appear to go on to produce sus cells, while GBCs expressing mammalian achaete-scute homologue-1 (Mash1) predominantly produce OSNs. Cells expressing both Sox2 and
Mash1 can be found, likely representing a common progenitor of these two pathways (Gokoffski, et al. 2011) (Figure 1.3b). GBCs sequentially express Mash1 and neurogenin1 (Ngn1) along their developmental path to producing neurons (Cau, et al. 2002; Cau, et al. 1997). As they differentiate into neurons, they express GAP43 and begin to sprout dendritic and axonal processes. Productive binding of O/E transcription factors to their binding sites on mature OSN protein promoters leads to the transcription of mature OSN genes including ACIII, Golf, OcNC, OMP, and an OR, which are associated with differentiation into OSNs (Wang and Reed 1993; Wang, et al. 1993; Wang, et al. 1997). Recent work in our laboratory suggests that the zinc-finger protein OAZ may act as a molecular switch that enables O/E transcription factor activation (Cheng and Reed 2007; Tsai and Reed 1997; Tsai and Reed 1998). Below the basal lamina reside the Bowman’s glands, whose ducts traverse the epithelium to secrete the protective mucous that lines the apical surface of the OE. Given the capacity for self-renewal of the OE, a stem cell population must exist, though it is unclear at this point whether such a cell resides among the HBC or GBC population (Calof, et al. 1998; Crews and Hunter 1994; Mackay-Sim 2010).

**OE development**

By mechanisms that are incompletely understood, but which likely are promoted by retinoic acid, fibroblast growth factor (FGF), and bone morphogenic protein (BMP) signaling, the olfactory placode develops from a thickening of the ectoderm at E9 (Schlosser 2006). Continued expression of FGF appears to promote an olfactory sensory fate, whereas BMP signaling appears to drive the developing tissue toward a respiratory
fate (Maier, et al. 2010). Continued expansion of this tissue leads to invagination to form the olfactory pits. Labeling for proliferating cells indicates bursts of cellular expansion, which can lead to bulges of epithelium up to 40 cells thick (Murdoch and Roskams 2007). Cellular expansion is driven by the olfactory placode precursor cells (OPPs), which initially divide predominantly at the apical surface, but transition to the basal compartment around E12 (Cuschieri and Bannister 1975). These OPPs express sox2, 
ascl1, and hes1, bearing resemblance to the GBCs of the mature OE (Packard, et al. 2011). The development of neurons proceeds along a well-defined pathway, which is first established by the expression of Mash1, a neuronal-permissive transcription factor. Mice lacking functional Mash1 have markedly diminished neuronal generation in the OE (Murray, et al. 2003). Mash1 cells give rise to immediate neuronal precursors that express neurogenin1 (Ngn1), which give rise to GAP43-expressing immature neurons that in turn give rise to mature OSNs, which first appear around E12.5 (Cau, et al. 2002; Cau, et al. 1997).

The embryological lineage of the keratin-expressing sus cells and HBCs is less well established. These cell types appear to fully develop at later embryonic stages, with both cell types apparent by E15 (Cau, et al. 1997; Packard, et al. 2011). Recent evidence suggests that these cells may also be derived from the OPPs. Persistent expression of sox2 may lead to development into sus cells (Gokoffski, et al. 2011), whereas activation of p63 may lead to the development of HBCs (Packard, et al. 2011), though definitive lineage analysis of OPPs has yet to be performed to confirm the latter suggestion. These observations suggest that OPPs may serve as a common progenitor that produce all cell types of the OE, including both GBCs and HBCs. It is unclear whether such a multipotent
progenitor resides among the GBCs in the adult, or whether HBCs are the only required multipotent stem cell. Whatever the relationship between these cells, it is clear that multiple steps are involved in determining differentiation, and a number of extrinsic signaling pathways have been implicated in controlling these processes.

**Extrinsic signaling**

During embryogenesis, the growth and differentiation of various cell types and tissues are shaped by a vast array of cell signaling pathways, including notch, wnt, fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor (TGFα and TGFβ). At the olfactory placode stage, FGF and EGF are important in stimulating neurogenesis and promote the creation and sustenance of a stem cell niche. The neural lineage is established by Fgf8, which is expressed highly at the rim of the invaginating pit (Kawauchi, et al. 2004). Once the mature OE is established, Fgf2 is important in maintaining the stem cell niche. EGF and TGF stimulate HBC proliferation (Mahanthappa and Schwarting 1993).

Expansion of the OE is reigned in during embryogenesis by TGFβsuperfamily signaling, especially members of the BMP and activin families. (Gokoffski, et al. 2011; Shou, et al. 1999). The ability of these molecules to inhibit neurogenesis has been well-documented in vitro. Neurogenesis in OE primary culture is inhibited by high dose BMP2, BMP4, and BMP7. These molecules act specifically on the Mash1 + progenitor population (Shou, et al. 1999). GDF11 also inhibits neurogenesis in vitro, but acts to prevent INP replication (Lander, et al. 2009) Two members of the activin family, GDF11 and Activin βB, have demonstrated the ability to inhibit neurogenesis in vivo. It is
believed that GDF11 acts upon immediate neuronal precursors, whereas activin βB acts upon a multipotent progenitor cell upstream of the INPs (Gokoffski, et al. 2011). Further, GDF11 knockout mice show a thickening of the OE, with overproduction of neurons and an expansion of the neuronal layer (Wu, et al. 2003). Conversely, knockout of follistatin (FST), a potent inhibitor of GDF11 and other TGFβ molecules, leads to a dramatically thinned OE, with negligible neurogenesis (Gokoffski, et al. 2011; Wu, et al. 2003). All of these findings underscore the ability of this class of molecules to regulate neurogenesis in the olfactory epithelium. However, despite these advances in understanding how extrinsic signaling pathways drive and tailor neurogenesis pathways during embryogenesis, it is unclear whether these same pathways are utilized during homeostasis and regeneration of the postnatal OE.

**OE Regeneration**

There is limited capacity for regeneration within the central nervous system of adult mammals. In most areas of the brain, destroyed neurons are not replaced, leading to devastating consequences following brain injury. This has led to an interest in understanding the mechanisms controlling neurogenesis in the few areas in which it takes place, for these areas contribute much to maintaining plasticity and regenerative potential in the CNS. These areas include the dentate gyrus of the hippocampus, the subventricular zone, and the olfactory epithelium. Of these areas, the olfactory epithelium has demonstrated the most prolific neurogenic potential. Likely due to their exposure to environmental insults, there is a continual proliferation of basal cells in the OE, which mature into OSNs (Mackay-Sim and Kittel 1991a). The lifespan of OSNs appears to be
limited. While some neurons have been demonstrated to live as long as a year in mice living in purified air environments (Hinds, et al. 1984), labeled neurons usually do not survive nearly so long in the OE (Graziadei and Graziadei 1979; Mackay-Sim and Kittel 1991b). Some have even suggested that, given potential for infectious inoculation of the brain via the OE (Owen, et al. 2009), OSNs may be predisposed to apoptosis as a means of immunological defense (Harris, et al. 2009). Irrespective of the baseline propensity for apoptosis and neurogenesis, the OE has demonstrated a profound ability to regenerate and repopulate all cell types after various experimental lesioning paradigms.

Several different methodologies have been utilized to conduct lesion experiments of the OE, utilizing the accessible location of the OE and OB. Surgical ablation of OSN axons by olfactory nerve transection or olfactory bulbectomy leads to a selective loss of OSNs, while sparing other OE cell types (Carr and Farbman 1992; Costanzo 1985). This leads to an increase of basal cell proliferation, and generation of sufficient neurons to nearly reconstitute the OE. In the case of olfactory nerve transection, this leads to OE repair, with new neurons innervating targets in the OB (Costanzo 1984). However, because the synaptic target is removed during bulbectomy, full recovery is not possible (Gordon, et al. 1995). In fact, in mice older than 13 days, OSN axons fail to penetrate into the OB cavity due to the formation of glial scar tissue (Evers, et al. 1996).

More extensive lesions can be produced by the introduction of toxic agents to the apical surface of the OE, which can lead to destruction of more cell types, including sus cells and some basal cell populations. Under most conditions, HBCs are spared. ZnS04 (Matulionis 1975), Triton-X-100, and MeBr gas (Schwob, et al. 1995) have all been used as toxic agents for this purpose. Similar effects can be obtained by the injection of the
anti-thyroid drug methimazole (Bergman, et al. 2002). Even under these harsh conditions, the epithelium can be reconstituted to pre-lesioned status within a few weeks, with the recovery preserving the normal zonal expression pattern of OSNs (Iwema, et al. 2004). For lesion experiments for which reinnervation of the olfactory bulb is possible, complete functional recovery appears to occur, suggesting appropriate rewiring (Harding and Wright 1979; Hurtt, et al. 1988; Yee and Costanzo 1995).

In this thesis, experiments are described that study the process of neurogenesis in mouse OE. By utilizing genetically-modified mice, I was able to robustly-express follistatin in adult mice, allowing the study of TGFβ knockdown in adult animals. Despite effective induction of expression, no effects on neurogenesis could be observed, bringing into question the importance of this signaling pathway in modulating neurogenesis in the adult. A separate line of investigation utilized a lineage-tracing paradigm to study the dynamics of GBC differentiation. Postnatal labeling of Mash1-expressing cells marks a transient amplifying cell that gives rise to neurons. These labeled cells do not amplify in response to olfactory bullectomy. Conversely, labeling of Mash1-expressing cells in utero marks cells that differentiate into a variety of cell types, most notably HBCs. Together, these observations suggest that the postnatal Mash1-expressing GBCs have distinct characteristics compared to the embryonic Mash1-expressing OPPs.
Figure 1.1. Organization of the Olfactory System

(A) Schematic sagittal view of the mouse olfactory system. Volatile odorants enter the nasal passage (NP) and interact with OSNs in the main olfactory epithelium (MOE), which send their axons through the olfactory nerve (ON) to the main olfactory bulb (MOB). Pheromone detection occurs predominantly at the vomeronasal organ (VNO), which sends its signals through the vomeronasal nerve (VN) to the accessory olfactory bulb (AOB). (DeMaria and Ngai 2010)

(B) Schematic representation of the path from odorant molecule to the brain. 1. After entering the nasal cavity, odorants interact with odorant receptors on the cilia of OSNs, located in the olfactory epithelium. 2. Odorant binding leads to the propagation of action potentials down OSN axons to the olfactory bulb, 3. where they synapse onto second order neurons.

(http://www.nobelprize.org/nobel_prizes/medicine/laureates/2004/illpres/2_olfactory.htm)
Figure 1.2 Zonal expression of olfactory receptors

The expression pattern of any given OR is restricted to a particular domain along the dorsal-ventral axis of the olfactory epithelium. This arrangement is carried into the bulb, where the OSN axons converge into glomeruli that are in a similar dorsal to ventral arrangement. (Mori, et al. 2000)
**Figure 1.3 Cellular components of the olfactory epithelium**

(A) Schematic representation of OE cell types. At the apical surface of the epithelium, the sustentacular cells are found (yellow). These cells extend foot processes that span the epithelium and contact the basal lamina. Just below the sustentacular cells are the OMP-expressing mature OSNs (purple), which send an apical dendrite above the sus cells, and elaborate cilia above the epithelium in the nasal mucus. OSNs span the neuronal layer. Within this layer are also found GAP43-expressing immature neurons (light blue). Below the neuronal layer is the basal layer, which is home to two cell types. The HBCs (green) are flat, keratin-expressing cells found at the most basal extreme of the OE, adherent to the basal lamina. Just above the HBCs are GBCs (dark blue), a heterogeneous population of round cells. These cells may express Mash1, Sox2, or a combination of both. (Duggan and Ngai 2007)

(B) Schematic diagram demonstrating the Differentiation Pathways of OPPs. During embryogenesis, a population of cells exists that resembles adult GBCs. A subpopulation of these cells co-express Mash1 and Sox2. Expression of Mash1 is permissive for these cells to differentiate along the neuronal lineage, producing OSNs after a brief period of transient amplification. Recent evidence suggests that persistent expression of Sox2, may instead lead to these cells differentiating into sustentacular cells (Gokoffski, et al. 2011).
Chapter II: Inhibition of TGFβ Signaling Does Not Alter Adult Neurogenesis
Introduction

Animals are complex organisms comprised of many trillions of cells. In adults, the majority of these cells are highly specialized and terminally differentiated. These differentiated cells have exited the cell cycle, and developed specialized morphologies to carry out their function through unique profiles of gene expression. Before tissues arrive at this state – during embryogenesis – there are abundant actively dividing progenitor cell populations that must differentiate to form the mature cell types of the tissue. Given the high fidelity with which cell types and tissues are produced across organisms within a species, it is clear that this is a highly regulated process in which many different signaling pathways and transcription factors play an important role.

One class of molecules, the TGFβ family, has been identified as a negative regulator of tissue growth (Massague and Gomis 2006). Mature muscle cells secrete myostatin, a member of this family, to inhibit proliferation of immature muscle cells, allowing the tissue to achieve its normal size (Lee and McPherron 1999; McPherron, et al. 1997). GDF11, a close relative of myostatin, has been shown to provide a similar type of feedback in the growing embryonic OE. In particular, it has been demonstrated that mice lacking functional GDF11 will develop a thickened OE with an expanded neuronal layer. In contrast, knocking out follistatin, a potent inhibitor of TGFβ signaling, leads to a dramatically-thinned OE, with minimal neuronal development. (Gokoffski, et al. 2011; Wu, et al. 2003).

The olfactory epithelium is in direct contact with the external environment, and consequently is continually exposed to injury. As a consequence, OSNs die and are replaced throughout life. Given its role in fine-tuning the regenerative process during
embryogenesis, it is logical to suppose the TGFβ family may play a similar role in the adult. However, experiments that utilized a germline knockout of the GDF11 gene could not examine the role of this factor in adult tissue since these animals do not survive past the neonatal period. We sought to investigate the role of this family of molecules in regulating neurogenesis in the adult by utilizing strategies to manipulate this signaling pathway exclusively in the olfactory epithelium, preventing the lethality associated with abolishing TGFβ signaling in other tissues. Conditional knockdown strategies were employed, utilizing Cre/Lox technology. Additionally, genetically-modified mice were developed to express TGFβ molecules or a selective inhibitor of this signaling pathway. We were able to temporally induce high levels of the TGFβ inhibitor follistatin within the olfactory epithelium. Prolonged exposure to this inhibitory molecule did not produce noticeable effects on the olfactory epithelium, raising doubts that this system retains its important regulation of neurogenesis in the postnatal OE.

Methods

Mouse lines, genotyping, and TRE induction

TRE-Follistatin, TRE-Myostatin, GDF11 flox, and GDF11null mice were kindly provided by Dr. Se-Jin Lee. OMP-Cre mice were obtained from Dr. Peter Mombaerts. Cyp2g1-rTA mice were provided by Dr. Andrew Lane. Genotyping strategy for GDF11flox and GDF11 null mice is provided in Figure 2.1a. OMP-Cre mice were genotyped by PCR amplification of the Cre-recombinase gene product, utilizing the following primers: forward primer: 5' - AACATGCTTCATCGTCCGGTCCGCTGC -
3', reverse primer, 5' - GACGGAATCCATCGCTCGACCAGTTTA - 3'. TRE-Follistatin and TRE-Myostatin mice were genotyped by PCR amplification of the SV40 region of the construct utilized to generate the mice. The genotyping primers utilized were: forward primer, 5’- TTGCCTCCTGCTGCTGCTGC-3’, reverse primer, 5’-TTTTTCCCAGGTCCACAGTCCACG-3’. Cyp2g1-rtTA mice were genotyped by PCR amplification of the rtTA gene, utilizing the following primers: 5’-AACCCGTAACACTCGCCCCAGAAG-3’, reverse primer, 5’-CGCAACC TAAAGTAAAATGCCCC-3’. For induction of TRE genes, mice were fed a grain-based diet with Doxycycline added (2gm/kg, Bio-Serve) for at least 30 days unless otherwise specified.

**BrdU Injection and Tissue Preparation**

Where indicated, mice were injected intraperitoneally with 0.5 mg bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) per 10g body weight 4 hours before sacrifice. Mice were deeply anesthetized with xylaket (Sigma, St. Louis, MO), and perfused with ice cold 4% PFA in PBS. Heads were prepared as previously described (Lewcock and Reed 2004), and post-fixed in 4%PFA for 30 min – overnight at 4 °C. Heads were switched to a solution of 30% sucrose, 250mM EDTA in PBS and incubated at 4 °C for 24hr – 72 hr. Heads were flash frozen in O.C.T. (Tissue-Tek, Torrance, CA) over dry ice or liquid nitrogen, and stored at -80 °C before sectioning. Tissue sections were cut at 8 – 20 µm and stored at -80 °C.
**In Situ Hybridization, Immunohistochemistry, and microscopy**

In situ hybridization was performed as previously described (Wang, et al. 2004), utilizing the following RNA probes: hFollistatin (nucleotide 1-1034 of NM_013409), GDF11 (nucleotide 341 -736 of NM_10272.1), GAP43 (nucleotide 147 to 806 of NM_030553) and OMP (partial cDNA of NM_011010). For immunohistochemistry, the following primary antibodies were utilized: rabbit anti-hFollistatin (1:1000, NIDDK National Hormone and Peptide Program), and rat anti-BrdU (1:200, Abcam). Slides stained with anti-BrdU were treated with 3N HCl for 30 min before incubation with primary antibody. Fluorescent visualization of primary antibody was accomplished utilizing the following secondary antibodies: Alexa-488 or Alexa-546-conjugated species-specific IgG. Chromogenic visualization of primary antibodies was accomplished using horseradish peroxidase (HRP)-conjugated species-specific IgG (Vector Laboratories, Burlingame, CA) developed with the diaminobenzadine (DAB) substrate (Sigma, St. Louis, MO). Images were captured with a LSM510 confocal microscope or Zeiss Axioplan.

**RNA isolation and realtime-PCR**

Mice were euthanized with carbon dioxide, and olfactory tissue was harvested and ground in ice cold Trizol (Invitrogen) in a Potter-Elvehjem tissue grinder. RNA was extracted according to Invitrogen’s Trizol protocol. 1 ug of resuspended RNA was treated with DNase, and reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the Invitrogen protocol. Oligo dT primers were used to prime the reaction. Real time PCR was performed on an Applied Biosystems Step One Real-Time PCR machine utilizing the following primers: GDF11: forward primer, 5' -
Results

A Conditional Approach to GDF11 knock-down in mouse OE

The role of TGFβ signaling in modulating neurogenesis in mouse OE has thus far been studied primarily in the embryo. The role played in modulating neurogenesis has been demonstrated for activin βB and GDF11 utilizing gene knockdown strategies in mice (Gokoffski, et al. 2011; Wu, et al. 2003). However, these mice do not survive postnatally due to extra-olfactory manifestations of TGFβ knockdown, hampering the study of this system in adult mice. To circumvent these difficulties, we developed a strategy utilizing the Cre/LoxP system to conditionally knockdown GDF11 signaling in the olfactory epithelium. Given that GDF11 has been suggested to be produced predominantly by
OSNs (Wu, et al. 2003), we utilized previously developed mice expressing Cre recombinase from the endogenous OMP locus. Given the high efficiency of activation of the OMP-cre locus (Li, et al. 2004), GDF11 expression could be eliminated in mature OSNs with loxP sites inserted at both GDF11 alleles (Figure 2.1a). To determine the efficacy of gene knockdown in the OE, real time RT-PCR was performed on RNA isolated from whole preparations of OE from adult GDF11flox/flox ; OMP-Cre mice. No reduction of RNA message levels was found in conditional knockdown mice when compared with littermate control GDF11 flox/flox mice lacking the OMP-Cre allele (1.1 +/- 0.46 vs 1.2 +/- 0.24, respectively) (Figure 2.1b).

It is possible that expression of GDF11 remained high across the OE due to ineffective recombination of the GDF11 flox allele. To address this, we examined GDF11 expression levels in mice heterozygous at the GDF11 allele with GDF11flox/null. The GDF11 null allele has been previously demonstrated to not produce functional GDF11 protein, and is neonatal-lethal in the homozygous state. With this strategy, only a single recombination event is needed in each neuron to knock out GDF11 expression. However, these mice also demonstrated similarly normal levels of GDF11 expression (data not shown.)

Real-time PCR is useful for detecting global levels of expression across a tissue. In order to determine whether expression of GDF11 was reduced in discrete areas or cell types within the OE, we performed in situ hybridization to detect GDF11 message on coronal tissue sections. This methodology also failed to reveal a difference in expression between the GDF11 flox/flox ; OMP-Cre mice and their non-cre containing littermates.
(Figure 2.1c) Given that this conditional approach did not appear to achieve the desired result of knocking down GDF11, further analysis of these mice was not pursued.

**A Strategy to Inducibly Express a GDF11 analog in the OE**

Given that germline knockdown of GDF11 leads to excessive neurogenesis and thickening of the OE in the embryo, overexpression of GDF11 should conversely lead to a decrease in neurogenesis and OE atrophy. Utilizing the tet/rtTA system, we developed a method to inducibly express myostatin in the mouse OE. Myostatin was substituted for GDF11 because the TRE-Myostatin mouse had already been generated by Dr. Se-Jin Lee, and the active protein formed by this construct is nearly identical to that of GDF11 (McPherron, et al. 2009). Previous work in our laboratory demonstrated that, upon doxycycline administration, the cyp2g1-rtTA can cause robust activation of a transgene equipped with a TRE promoter in the sus cells of the OE (Figure 2.2a-b). Robust expression and secretion of TNF-α was observed in cyp2g1-rtTA ; TRE-TNF-α mice shortly after the administration of food containing 2g/kg doxycycline. (Lane, et al. 2010). Secretion takes place at the foot processes, located in the basal zone of the OE. This location is ideal for the secretion of a TGF-β molecule presumed to inhibit neurogenesis through cell cycle inhibition of progenitor cells.

To determine whether this system would produce elevated levels of myostatin in the mouse OE, adult mice were exposed to doxycycline in their food for > 30 days, after which RNA from whole OE was purified and quantified by real time RT-PCR. These preliminary results did not demonstrate an increase in myostatin mRNA levels compared to littermates lacking the TRE-myostatin construct. Since silencing of TRE-containing
constructs has been suggested to occur in some lines with age (Takiguchi, et al. 2013), the experiments were repeated with mice being started on Doxycycline as early as during embryonic development. Whether doxycycline exposure began at P0, P19, or E0 (i.e. parents were started on doxycycline before mating), no increases in myostatin message levels were observed in any case. Figure 2.2c shows combined real time RT-PCR results from these mice with littermate controls, whose expression levels were 1.2 +/- 1.0 (st. dev) (n = 8) vs. 1.0 +/- 0.7 (st. dev.) (n = 4) respectively. By comparison, message levels in skeletal muscle were 16.9 +/- 7.0 (n =3). Given the robust activation seen with other TRE-driven genes in this system, such as TRE-TNFα or TRE-GFP (Lane, et al. 2010), this failure of expression is most likely due to silencing of the TRE transgene. Poor expression of this particular TRE construct had been previously observed (Se-Jin Lee, unpublished). Given the inability to express the TRE-myostatin gene, an alternative strategy was developed to alter TGFβ signaling in the adult OE.

Robust Inducible-Expression of Follistatin in the OE

Follistatin irreversibly binds and blocks activity of TGFβ family members. It is endogenously expressed and is believed to be important in fine-tuning TGFβ signaling in vivo. The importance of this molecule in this pathway is demonstrated in follistatin knockout mice, which demonstrate profound inhibition of neurogenesis in the OE (Gokoffski, et al. 2011; Wu, et al. 2003). If TGFβ signaling is equally important in controlling neurogenesis in the adult, inducing expression of follistatin should lead excessive neurogenesis and expansion of the OE. We developed a strategy to induce follistatin expression employing a similar strategy as had been attempted with myostatin,
substituting the TRE-hFollistatin transgene (Figure 2.3a-b). In contrast to the TRE-myostatin transgene, we saw robust induction of follistatin expression in these mice after administration of doxycycline (dox). Before administration of dox-containing food, message levels of the human follistatin transgene were either undetectable or found at very low levels. By the third day of a dox exposure, message levels increased to >-50,000-X the leaky expression levels (53,800 +/- 10,100), and rose another order of magnitude by 30 days of constant exposure to doxycycline (426,000 +/- 236,000) (Figure 2.3c). These day 30 levels were 10X higher than the OMP message, 9.8 +/- 5.5 vs. 1.2 +/- 0.27 (Figure 3.1d), which is highly expressed by all mature neurons, with expression levels on par with those of the housekeeping gene GAPDH.

We next sought to examine the expression pattern of the follistatin transgene. In situ hybridization analysis demonstrates that follistatin is highly expressed in a subset of sustentacular cells (Figure 2.4a-b). It is unclear why transgene expression is non-uniform, but this pattern has been previously observed with this cyp2g1 rtTA construct (Lane, et al. 2010). The robust mRNA levels observed for the induced follistatin transgene suggested that elevated levels of follistatin protein would be present in the sustentacular cells. Antibody labeling for follistatin demonstrates that these high message levels are translated into protein (Figure 2.4c) and distributed within the cell in a manner consistent with its eventual secretion.

**Prolonged Exposure to Follistatin does not Affect Neurogenesis**

Knockout of follistatin in the embryo leads to a profound inhibition of neurogenesis and dramatic thinning of the OE (Gokoffski, et al. 2011; Wu, et al. 2003). If similar
mechanisms are at play in the adult OE, overexpression of follistatin should lead to an increase in neurogenesis, resulting in a thickening of the OE, with an expansion of the OSN compartment. Expansion of the neuronal population should lead to an increase in message level of neuronal components. We therefore performed real time RT-PCR of mouse OE 30 days after continuous exposure to induced follistatin expression. There were no observable alterations in the thickness of the OE in these mice. No change in expression of the mature OSN transcript OMP, GBC transcript Mash1, or immediate neuronal precursor transcript Ngn1 were seen when compared to mice of identical genotypes not exposed to doxycycline (Figure 2.5a). Given that follistatin is not uniformly expressed, it is possible that regional changes in neurogenesis may not be sufficient to alter gene expression after mRNA isolation from the crude nasal tissue. To test this, mice fed doxycycline for 30 days were fixed and tissue sections were assessed for expression levels by in situ hybridization for OMP, GAP43, and Follistatin. There was no observable difference in the thickness of OE, or in the expression pattern of GAP43 or OMP when compared to similarly-treated mice lacking the TRE-hFollistatin transgene.

One expected consequence of follistatin-induced alteration of OE dynamics is a modulation of proliferation of GBC progenitors. The effects of prolonged exposure to follistatin on proliferation were also assessed by quantifying the number of cells that incorporated brdU. Mice were treated for > 30 days of doxycycline, then injected with brdU 4 hours before sacrifice. Staining for proliferating cells did not reveal any increase in their number in the hFollistatin-expressing mice (Figure 2.6).
Discussion

TGFβ signaling has been demonstrated to be an important regulator of neurogenesis in the developing OE. Given the continual generation of neurons that takes place in the OE throughout life, it is reasonable to suppose TGFβ signaling plays a role there as well. Multiple strategies were employed to alter TGFβ signaling in the adult. The first was to employ a conditional knockout approach. Previous work had suggested that GDF11 was produced by OSNs. Utilizing the mature marker OMP to drive cre expression seemed a reasonable approach to knock down expression in an OE-specific manner. However, both immature and mature neurons have been implicated in the production of GDF11 (Wu, et al. 2003). It is possible that production in the OE was not diminished in the conditional knockout due to the persistent expression of GDF11 in other cell types, including immature neurons.

In addition to the conditional knockdown approach, an inducible inhibitor approach was attempted to block TGFβ signaling in the OE. However, despite robust expression of FST message and protein, no effects on neurogenesis could be demonstrated. This could be due to a number of factors. The first possibility is that, despite being produced at high levels, the FST was not properly processed and delivered to the area of the tissue that was needed. Processing of the protein should not have been an issue in this case, as a variant of follistatin that was utilized that did not require additional steps to become active. It also seems unlikely that the follistatin was not being appropriately delivered. Sustentacular cells have a highly developed secretory apparatus, and they secrete into the basal layer of the OE at their foot processes. This is presumably
where much of the regulation of neurogenesis is taking place, and therefore an ideal place for FST to be delivered. The previous success of this sustentacular cell-specific induction system to direct effective production and secretion of TNF-α (Lane, et al. 2010) further suggests that the secretory apparatus likely did not constrain follistatin function.

A second possible reason for the failure to discern a phenotype in the inducible follistatin mice is that the patchy expression pattern may have made a phenotype difficult to detect. Any small local effects in the number of proliferating cells or in the number of neurons generated may have been been obscured by the large areas that did not have follistatin-expressing cells in the area. An attempt was made to analyze the data taking this into account, but it is difficult to define the “area of influence” that could be caused by follistatin-secreting cells. The absence of insight into the range of diffusion or action of the secreted follistatin made this approach complex.

It is also possible that the conditions that were employed were not optimized to reveal a phenotype. Our experimental designs were based on the hypothesis that prolonged exposure to TGFβ inhibitor would be most likely to disrupt neurogenesis in a sufficiently significant way to have a demonstrable phenotype. However, it is possible that there is a transient alteration in neurogenesis that occurs soon after the initiation of follistatin induction that was missed by our detection methods. Furthermore, it is possible that the correct outcomes were not assessed. The primary outcome of interest was neurogenesis, leading to the analysis of neurons and proliferation. However, recent evidence suggests that in the presence of strong inhibition of the activin family, the most notable outcome was an increase in sustentacular cell production (Gokoffski, et al. 2011). In an effort to examine this question we assessed expression of Sox2 (a sustentacular cell
marker) in a subset of samples by QPCR and antibody staining, and did not observe any changes in Sox2 expression in the OE of mice exposed to high levels of follistatin. Finally, it is possible that no phenotype was observed because TGFβ signaling does not significantly contribute to the dynamics of neurogenesis and cellular homeostasis in the adult OE. In this case, it is curious that these molecules continue to be expressed through life. However, it is possible that the process of tissue maintenance is different and requires different signaling strategies than are utilized in the developing embryo. This possibility would corroborate the outcome of the next chapter in this thesis, both of which suggest that the cell types and signaling pathways utilized during embryogenesis may be distinct from those utilized in maintenance of the post-natal OE.
Figure 2.1 Approach to Conditionally Knock Down GDF11 in OE

(A) Schematic representation of the wild type GDF11 allele (top), GDF11 flox allele, with loxP sites (gray triangles) inserted before exon 2 and after exon 3 (middle), and the GDF11 null allele (bottom), in which a neo cassette (white box) is inserted into exon 3, rendering the protein non-functional. The letters A-D indicate the position of primers used to genotype these mice. Primer sequences are: A: 5'
AAGGCTTGGGAACGAGCAGCAAG - 3', B: 5'- AGGTATGGTTAGGTGTGAGGAG-3',
C: 5'-AATGTCTGGTGGGAGCGCCGTAAAC-3', D: 5'-
TGGATGTGAATGTGTCGAG-3'. PCR of A+B gives 359bp product in Wt or null allele, 393bp product in floxed allele. PCR of C+D gives 386bp product in null allele, no product in other alleles.

(B) GDF11 RNA expression levels are unaltered by conditional knockdown strategy. Relative mRNA message level, as measured by real-time RT-PCR, demonstrate no difference between conditional knockdown mice (GDF11 flox/flox ; OMP-Cre ) (white bar) and control mice (GDF11 flox/flox mice ; OMP +/- ) (black bar). Values are corrected against GAPDH levels, and presented as mean +/- standard deviation. (n=2 for each group)

(D) GDF11 expression pattern is unaltered by conditional knockdown strategy. There is no distinguishable difference in the pattern of expression between the control (GDF11 flox/flox; OMP +/-) and conditional knockdown (GDF11 flox/flox ; OMP-Cre/+ ) mice. Sense probe control is shown. Scale bars = 10 µm.
A

1 kb

GDF11^+  

GDF11^lox

GDF11^KO

B

Relative mRNA Expression

0.0  0.5  1.0  1.5  2.0

GDF11^floX/flox  GDF11^floX/flox

GDF11^floX/flox ; OMP^+/+

GDF11^floX/flox ; OMP^Cre/+  (sense probe)

C

GDF11^floX/flox ; OMP^+/+

GDF11^floX/flox ; OMP^Cre/+  (sense probe)
Figure 2.2 Doxycyclin-induced expression is not observed in TRE-Myostatin; cyp2g1-rtTA mice.

(A) Schematic representation of the genetic strategy utilized to induce myostatin expression in sustentacular cells. In sustentacular cells expressing the cyp2g1-rtTA gene, administration of doxycycline will lead to activation of the TRE promoter, driving expression of myostatin.

(B) Schematic representation of a mouse OE. A sustentacular cell is highlighted in yellow, to depict the cell type that will be expressing myostatin.

(C) Myostatin mRNA expression levels in whole OE are not increased after doxycycline induction. All mice were exposed to doxycycline for at least 30 days before sacrifice. Expression levels are unchanged in TRE-myostatin; cyp2g1-rtTA mice compared to control (cyp2g1-rtTA mice). Expression levels in skeletal muscle are shown for comparison, which are 16 X higher than levels observed in OE. Values are corrected against GAPDH levels, and presented as mean +/- standard deviation. (n=4 for control OE, n = 8 for TRE-myostatin OE, n = 3 for skeletal muscle).
A

cyp2g1-rtTA

TRE-myostatin

Dox

TRE

myo

B

C

Relative mRNA Expression

OE Control Mouse
OE cyp2g1-rtTA TRE-Myostatin
Skeletal Muscle Control Mouse

25.0
20.0
15.0
10.0
5.0
0.0
Figure 2.3 Robust Induction of Follistatin levels in OE

(A) Schematic representation of the genetic strategy utilized to induce hFollistatin expression in sustentacular cells. In sustentacular cells expressing the cyp2g1-rtTA gene, administration of doxycycline will lead to activation of the TRE promoter, driving expression of hFollistatin.

(B) Schematic representation of a mouse OE. A sustentacular cell is highlighted in yellow, to depict the cell type that will be expressing follistatin.

(C) Follistatin levels are rapidly and robustly increased in mouse OE after doxycycline administration. By the third day of doxycycline administration, follistatin levels have risen to 50,000 X the leaky baseline levels. These levels rise another order of magnitude by day 30 of continuous doxycycline administration. Values depicted are mean (of 3 mice per time point) +/- standard deviation.

(D) Comparison of follistatin levels to the highly expressed OMP transcript. The expression level of follistain message is 10 fold higher than OMP levels by 30 days of continuous doxycycline administration. Values depicted are mean (of 3 mice) +/- standard deviation.
Relative Follistatin Expression Level

Days of Doxycycline Induction

Relative mRNA Expression Levels

hFoll OMP hFoll OMP

No Dox 30 Days Dox

cyp2g1 Promoter rTA
TRE-hFollistatin
Dox
TRE hFoll
TRE hFoll

Cyp2g1 Promoter
rtTA
TRE-hFollistatin
rtTA
Dox
TRE hFoll
TRE hFoll

C

D
Figure 2.4 Follistatin induction is nonuniform

(A) ISH on hemisection of mouse OE demonstrates that only a subset of sustentacular cells express the follistatin message. The distribution of follistatin-expressing cells does not appear to occur in a definitive pattern. Scale bar = 100 µm.

(B) High power view of follistatin message expression pattern. As expected, the cells expressing the construct demonstrate the signature wine-glass morphology of sustentacular cells. Scale bar = 25 µm.

(C) Follistatin protein is made in abundance by sustentacular cells. Anti-follistatin Antibody staining demonstrates labeling in a pattern that is similar to that seen by ISH. Scale bar = 25 µm.
Figure 2.5 Neurogenesis is not altered by long-term follistatin production

(A) Message levels of cells in the neuronal lineage are not affected by follistatin. RNA was harvested from the OE of adult TRE-follistatin; cyp2g1-rtTA mice before (black bars) or after (gray bars) administration of doxycycline for 30 days. Real time RT-PCR of OE did not demonstrate a difference in message levels of transit-amplifying neuronal precursors (Mash1), immediate neuronal precursors (Ngn1) or mature neurons (OMP). Values displayed as mean (of 3 mice) +/- standard deviation.

(B) Expression pattern of neuronal-lineage-specific messages is unchanged after long-term doxycycline induction. TRE-FST; cyp2g1-rtTA and control littermates (cyp2g1-rtTA) mice were exposed to doxycycline for at least 30 days. ISH demonstrates that follistatin expression is robustly induced, but the pattern of expression of GAP43, denoting immature neurons, and OMP, denoting mature neurons, is not altered by follistatin expression.
A

Relative Message Levels

(-) Dox  (+) Dox (30 Days)

Mash1  Ngn1  OMP

B

FST  GAP43  OMP

TRE-FST: cyp2g1-rTA
cyp2g1-rTA
Figure 2.6 Proliferation is unaltered after prolonged exposure to follistatin

(A) BrdU incorporation is unaltered in TRE-FST; cyp2g1-rtTA mice compared to littermate controls after 30 days of continuous administration of doxycycline. Scale bars = 10 µm.

(B) Quantification of BrdU labeled cells in TRE-FST; cyp2g1-rtTA mice compared to littermate controls. Values are listed as mean +/- standard deviation. n = 2 for each group.
A

TRE-FSTN; cyp2g1-rtTA

cyp2g1-rtTA

B

BrdU-Labeled cells/mm OE

cyp-rtTA  Tet-Foll ; cyp-rtTA
Chapter III: Comparative Analysis of Mash1-labeled Cell Dynamics
Introduction

There is limited capacity for the generation of neurons in the CNS of adult mammals, leading to significant mortality and morbidity in disease processes that result in central neuronal loss. The observation that a few areas of the adult CNS continually generate new neurons has provided hope that it may be possible to replace lost brain tissue. Understanding how these neurogenic regions are able to maintain this ability could lead to many therapeutic breakthroughs. Of the three areas that continuously generate neurons that synapse in the brain throughout life, the OE is perhaps the simplest and most easily accessible. Given the relative ease with which this tissue may be harvested, and its prolific capacity to heal itself, the OE could prove to be a perfect reservoir of neurogenic stem cells.

Although it is clear that the stem cell of the adult OE resides within the basal layer of that tissue, there is much debate over which of the two resident cell types is the true stem cell. Some researchers have toted the HBC as the true stem cell (Carter, et al. 2004; Mackay-Sim 2010). It is able to generate all other OE cell types both in vitro and in vivo, divides infrequently, and has demonstrated asymmetric cell division(Carter, et al. 2004; Leung, et al. 2007; Mackay-Sim and Kittel 1991a). In many respects, the properties of this cell resemble those of the presumed stem cells in the epidermis (Fuchs 2009). Others have suggested that the stem cell may reside within the population of GBCs, given their known ability to generate most cell types of the OE in vivo and in vitro (Calof, et al. 1998).
Until recently, much our understanding of the role of these two cell types has been inferred from either in vitro analysis of OE cell explants, transplantation studies of purified GBCs and HBCs, retroviral-labeling of OE cells, and colabeling studies of proliferating cells. While instructive, these methods suffer from either a lack of demonstrated relationship to regeneration in the native context (in vitro studies), or the potential for unnatural circumstances to influence the behavior of cells (transplantation). Further, colabeling studies would fail to capture the circumstances where a cell type down-regulates the protein used to label it before it can proliferate. This appears to be the case with HBCs, which are generally labeled with antibodies against keratin proteins, as these proteins are likely turned off before these cells reenter the cell cycle. A more definitive approach to ascertain the activity of a cell type can now be achieved through the use of cre-lox technology. Utilizing cre reporters that are specific for a given cell type, it is possible to irreversibly label a particular cell type and all of its subsequent progeny. Our lab previously utilized this strategy with a K5-creER construct to label HBCs (Leung, et al. 2007), demonstrating definitively that these cells have the capacity to serve as stem cells in response to chemical injury of the OE. However, these HBCs are rarely if ever activated during routine maintenance or in response to olfactory bulbectomy, where presumably activity of GBCs is sufficient to replace lost neurons. To further understand the role of GBCs in tissue maintenance and in response to bulbectomy, we utilized a similar labeling strategy to mark cells within the GBC population.

Here we utilize a Mash1-labeling strategy to study the dynamics of these GBCs. We show that labeling in the early postnatal mouse leads to the production of predominantly neuronal cells, and that a subset of these cells become established in the
epithelium and persist for at least 3 months. By combining this labeling strategy with olfactory bulbectomy, we demonstrate that there does not appear to be a stem cell labeled by these Mash1-expressing cells, given the failure of expansion and neurogenesis in response to this lesion paradigm. By contrast, we demonstrate that labeling of these Mash1+ cells during embryogenesis leads to the production of multiple different cell lineages, including sustentacular cells and HBCs in addition to neurons. These data suggest that the postnatal GBCs and embryonic OPPs are distinct cell types with unique characteristics.

**Methods**

**Experimental Animals and tamoxifen induction**

Mash1-CreER mice were produced by the collaborative efforts of our laboratory and the Jane Johnson laboratory. Details have been previously published (Kim, et al. 2011). Rosa-LacZ mice were purchased from Jackson Laboratory. K17-GFP mice were kindly provided by Dr. Pierre Coulombe. Rosa-tdtomato mice were kindly provided by Dr. Jeremy Nathans.

**Tamoxifen induction**

Postnatal mice were injected with 2mg tamoxifen (1mg/100µL) (Sigma, St. Louis, MO) per animal. Tamoxifen was weighed and dissolved fresh before each injection in 100%
ethanol, then suspended 1:10 in sunflower seed oil to reach the final concentration (Sigma, St. Louis, MO) by vortexing for 1 minute. For embryonic injections, tamoxifen was dissolved as above, and injected at a concentration of 6mg/40g. Given the difficulty of delivering after tamoxifen injection, fostering of pups was performed utilizing CD1 dams who had recently given birth. Where specified, 4OHT was substituted for tamoxifen, being prepared and injected as previously described (Badea, et al. 2003).

**BrdU Injection and Tissue Preparation**

Where indicated, mice were injected intraperitoneally with 0.5 mg bromodeoxyuridine (brdU) (Sigma, St. Louis, MO) per 10g body weight 2 hours before sacrifice. Mice were deeply anesthetized with xylaketa (Sigma, St. Louis, MO), and perfused with ice cold 4% PFA in PBS. Heads were prepared as previously described (Lewcock and Reed 2004), and post-fixed in 4%PFA for 30 min – overnight at 4 °C. Heads were switched to a solution of 30% sucrose, 250mM EDTA in PBS and incubated at 4 °C for 24hr – 72 hr. Heads were flash frozen in O.C.T. (Tissue-Tek, Torrance, CA) over dry ice or liquid nitrogen, and stored at -80 °C before sectioning. Tissue sections were cut at 8 – 20 µm and stored at -80 °C.

**Immunohistochemistry, X-gal staining, and microscopy**

Immunohistochemistry and X-gal staining were performed as previously described (Lewcock and Reed 2004), utilizing the following antibodies: rat anti-brdU (1:200, Abcam), anti GAP43 (1:500, Millipore), Anti O/E1 (1:1000, Connelly & Reed,
unpublished reagent). Slides stained with anti-brdU were treated with 3N HCl for 30 min before incubation with primary antibody. Fluorescent visualization of primary antibody was accomplished utilizing the following secondary antibodies: Alexa-488 or Alexa-546-conjugated species-specific IgG. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured on a Zeiss Axioplan or LSM510 confocal microscope.

**Olfactory bulbectomy**

Unilateral bulbectomy was performed on mice deeply anesthetized with xylaket diluted 1:10 in PBS at approximately 100µL/10g animal as described (Carr and Farbman 1992). Skull drilling was performed with a Northern Industrial Tools Mini Rotary Tool (NTE2100) equipped with a Dremel 107 engraving cutter bit. Stock Pasteur pipettes were used to remove olfactory bulb tissue with light vacuum suction. No packing was used to fill the empty cavity.

**Cell counting**

For each data point, the equivalent of a complete coronal section was counted for each mouse. This was either obtained by counting one complete coronal section, or hemisections from two different sections. Efforts were taken to count cells from similar areas of OE on the anteroposterior axis, judged by the amount of olfactory bulb present in each section. The total length of OE counted ranged from 9mm - 24mm. Data are presented as mean +/- standard deviation.
Results

Effective Labeling of GBCs in the neuronal lineage

In the OE, Mash-1 has been demonstrated to be expressed in a transit amplifying population of cells that predominantly gives rise to neurons (Beites, et al. 2005). However, lineage tracing of Mash1+ cells in the embryo has demonstrated that they also give rise to sustentacular cells (Gokoffski, et al. 2011). The authors suggest from these observations that this subpopulation of Mash1-expressing cells may represent a lineage restricted “stem cell” due to its multipotency. However, it has not been demonstrated that the Mash1-expressing cells present in the postnatal mouse retain this multipotency. We utilized the same lineage-tracing paradigm to observe the behavior of Mash1-labeled cells in postnatal olfactory epithelium. This knock-in mouse expresses CreER from the endogenous Mash1 locus. Induction with tamoxifen provided the ability to mark these cells in a temporally-controlled fashion (Figure 3.1a-b). Activated CreER can lead to recombination at a reporter locus, marking the Mash1-expressing cell and all subsequent Mash1-negative progeny to express the marker protein. Previous work with this labeling construct has demonstrated a specific labeling of Mash1-expressing cells (Kim, et al. 2007). Consistent with this study, we found that 3 days after tamoxifen injection of P7 mice, the majority of labeled cells resided in the basal zone, defined as within one cell diameter of the basal lamina (Figure 3.1c,e). At 3 days, 61% of labeled cells were found within the basal layer. By comparison, by 3 weeks after injection, only 11% of labeled cells remain within the basal zone. Accordingly, when sections of OE from mice
sacrificed at early time points are stained for the immature neuronal marker GAP43, the tdtomato-labeled cells are found predominantly below the GAP43 layer. In contrast, the labeled cells tend to be intermingled with, or more apical to, the immature neurons when examined at 3 weeks (Figure 3.1 c, d). These data suggest that cells corresponding to GBCs are labeled, and that these labeled cells move apically and the majority progress along a path consistent with neuronal differentiation. Accordingly, when OE tissue from mice sacrificed 3 days after labeling is immunostained for the early differentiation marker O/E-1, many tdtomato-labeled cells are observed that already express this protein, and are elaborating dendritic processes characteristic of OSN differentiation (Figure 3.1 g-h).

**Mash1-labeled cells give rise to a subset of resident OE cells**

To determine whether Mash1-expressing cells in the postnatal OE retained stem-like characteristics, we injected Mash1-CreER ; Rosa-tdtomato mice with a single 2mg dose of tamoxifen at age P7, and sacrificed at multiple time points from 3 days to 3 months in order to examine the dynamics of the Mash1-labeled cells. If labeling was restricted to a transiently-amplifying population of cells, one would expect that after a few cell divisions, all labeled cells would develop into neurons. These neurons would be expected to sustain themselves in the OE for a normal lifespan, previously estimated at 30-60 days. Further, one would expect a gradual decrease in the number of labeled cells over time. On the other hand, if a portion of the cells labeled had stem characteristics, we would expect to find evidence of multipotency, and the retention of cells with an undifferentiated, GBC phenotype. These cells might demonstrate active proliferation,
including the existence of clusters of expanding cells. We might also expect the quantity of labeled cells to remain constant, or even increase over time.

We found that the density of labeled cells in Mash1-CreER ; Rosa-tdtomato mice injected with tamoxifen at P7 was greatest within the first few weeks of labeling, and that it steadily decreased over time. Figure 3.2a shows a coronal section of labeling frequency at 3 days after injection, which is much greater than the labeled cell numbers observed at 2 months post-labeling (Figure 3.2b). Quantification of the labeled cell density shows an initial labeling density of 26.0 +/- 21.8 cells /mm OE (n=3 mice) when assessed 3 days after tamoxifen injection. The large standard deviation presumably reflects differences in labeling efficiency between mice. The labeling density remains high through 10 days (26.0 +/- 31.8 cells/mm OE (n=2)), but by 2 months, the density of labeled cells has decreased to 2.3 +/- 0.3 (n=2), and remains low at 3 months post labeling (3.5 +/- 0.5 cells/mm OE (n=2)) (Figure 3.2c). This pattern of cell dynamics suggests that only a fraction of the labeled cells remain in the OE after several months. While the majority of the labeled cells (61%) are found in the basal zone at 3 days, by 10 days the basal compartment contains only 22% of the labeled cells. However, a similar fraction remains at 2 and 3 months, with the percentage of labeled cells found in the basal zone being 15% and 16% at these time points, respectively. However, this does not necessarily indicate that these cells in the basal zone are GBCs. In fact, dendritic processes can clearly be seen in many of these cells. One way to determine whether these remaining basal zone cells are GBCs that retain proliferative capacity or post-mitotic neurons would be to examine how they respond to lesion of the OE.
**Mash1-labeled Cells Perish in response to Bulbectomy**

Although it is widely accepted that Mash1-expressing cells are primarily a transient-amplifying cell population that gives rise to neurons, it has been postulated that a subset of GBCs maintain stem cell character, and these cells are postulated to persistently express Mash1 (Gokoffski, et al. 2011). Additionally, it is known that robust neurogenesis takes place following olfactory bulbectomy, without activation of HBCs. It is believed that reconstitution of the epithelium under these conditions is mediated primarily by GBCs. To address this question, we performed unilateral olfactory bulbectomy one week after tamoxifen-induced OE labeling in Mash-creER; Rosa-ttdtomato mice. If a subset of cells within the labeled population were stem cell competent for proliferation, we would expect that, following bulbectomy, there would be a clonal expansion of such a cell, giving rise to multiple neurons, and potentially other cell types. If only a transient-amplifying cell was labeled, we would expect that the resident, post-mitotic labeled cells that had differentiated into neurons would be eliminated by bulbectomy. There does not appear to be any expansion of labeled cells at 5 or 10 days after lesion, and the labeled cells are essentially all eliminated by 30 days post-lesion (Figure 3.3). This is wholly consistent with the Mash1-CreER construct labeling cells demonstrating a transiently-amplifying behavior, without evidence of a stem cell population in this compartment.

**Mash1-labeled cells become restricted in their repertoire after birth**

GBCs have long been cited as a multipotent progenitor, as had been demonstrated in vitro and through transplantation experiments. However, there are few if any cells with
morphologies other than neuronal in the postnatally-labeled mice examined in the experiments reported here. Several explanations for this observation are possible. It could be that the specific labeling paradigm, using the Mash1-reporter, is not labeling the specific subtype of cells that demonstrate multipotency in published experiments. Alternatively, it is possible that the transplantation or culture conditions utilized in prior experiments were responsible for the observed behaviors. Specifically, the function of GBCs may have been altered by retroviral induction or transplantation. Additionally, recent experiments that demonstrated the potential of Mash1-labeled cells to give rise to sustentacular cells were performed at embryonic timepoints. To test whether differences may exist between the cell fates specified by Mash1-labeled cells pre-and postnatally, we injected pregnant Mash1-creER ; Rosa-tdtomato dams with tamoxifen at E12, a time when Mash1 expression is high in the developing mouse OE. We found that when labeled at this time, the cells clearly gave rise to sustentacular cells and HBCs in addition to neurons when examined at P14 (Figure 3.4). When these mice were crossed with a line expressing K17-GFP, cells were demonstrated that clearly expressed both reporter and GFP lining the basal lamina. These experiments definitively demonstrate for the first time that Mash1-labeled cells can give rise to cells with characteristics of HBCs.

Previous experiments in our laboratory demonstrated that HBCs remain quiescent during the neuronal turnover that takes place during olfactory bulbectomy in adult mice. Accordingly, examination of the OE 5 days after unilateral bulbectomy in a Mash-creER ; Rosa-tdtomato mouse tamoxifen injected at E12 reveals that the HBCs which were labeled at E12 remain quiescent in the OE (Figure 3.5). These HBCs are the predominant tdtomato cell type that remains on the lesioned side. As with the P7-labeled mice, there is
no evidence of clonal expansion of these labeled stem cells with HBC characteristics to repopulate the depleted OSNs.

Discussion

There exists considerable debate regarding which basal cell of the OE represents the tissue restricted “stem cell”. This debate is complicated by the correlation often made between the GBCs that are resident to the OE after birth, and the OPPs that proliferate during embryogenesis. Both of these cells types express Mash1. We utilized a lineage-tracing approach to compare the characteristics of GBCs and OPPs. We found that, as has been suggested in published work, cells that express Mash1 in the postnatal mouse behave like a transient amplifying cell. We found that within a few weeks of labeling there was a net loss of labeled cells in the tissue. Of course, caution should be maintained when interpreting quantitative comparisons across animals, especially considering the variability in labeling efficiency possible in these experiments. However, the trend toward a reduction of cells over time is clear and corroborates previous observations that the proliferative progenitors in the OE produce more cells than will eventually become stably incorporated into the tissue, with a considerable number of the neurons produced failing to make functional, stable connection to the olfactory bulb.

Despite the overall loss of cells over time, there were cells that remained in the OE for several months. That the majority of these cells reside in the neuronal zone and demonstrate neuronal morphology provides further evidence that, at least in purified air environments, olfactory neurons can have life spans that significantly exceed one month. Indeed, our observation that there is a dramatic reduction of labeled cells by one month
are also consistent with the observations that led to the frequently stated “one month” span of neurons. However, we also detected cells that resided within the basal zone at several months after labeling. Given that cells were observed that were adjacent to the basal lamina but were projecting axons and dendrites, it is likely that these cells were merely neurons occupying a basal position in the OE. However, it is possible that these are in fact cells that retain stem character. If this is the case, it is surprising that there was no proliferation, or even a quiescent persistence, of these cells in response to olfactory bulbectomy, a procedure that selectively kills mature neurons in the OE while sparing other cell types. That there was no clonal expansion or persistence observed in these cells makes it unlikely that they represent a cell with stem characteristics.

One of the confounding factors in the interpretation of labeling density in this work is the fact that we are studying young mice, in which the OE is still growing and expanding. This will naturally lead to a dilution of labeled cell density over time. It would be advantageous to label cells in older mice, when the OE is fully developed and this is no longer a consideration. However, it is difficult to adequately label cells utilizing the Mash1-CreER construct when injected later in life. This presumably reflects the lower abundance of target cells, increased metabolism of the tamoxifen in the target tissue and perhaps reduced targeting to the genomic locus. At the concentrations used in these experiments, we saw no labeling in mice labeled later than P21.

A final consideration in our experiments is that we may be labeling a specific fraction of the Mash1-expressing cells in the epithelium. For example, poor labeling efficiency may select for cells that are highly-expressing the Mash1-promoter. Such cells would be more likely to proceed along the pathway of neurogenesis, which may bias the
results toward neuronal fates among Mash1-labeled progeny. This seems unlikely given that labeling density was no greater in the embryo than in postnatal mice, and yet multipotency was observed in the embryonically-labeled mice. Another consideration is that the Mash1-CreER utilized is knocked into the endogenous locus. There does not appear to be any effects from only having a single copy of the Mash1 gene, but given that Mash1 knock out mice preferentially produce sustentacular cells at the expense of neurons, any reduced expression of Mash1 in our model system should in fact increase the probability that the reporter-labeled cells would proceed down a sustentacular differentiation pathway.

The studies reported here provide important new insights into the dynamics of olfactory epithelium regeneration and regulation. Specifically, a new Mash1-expressing cell type in the embryonic OE appears to give rise to two population of cells in the post-embryonic epithelium. A fraction of these cells are characteristic of the previously recognized Mash1-expressing GBCs while a subset of the embryonically-labeled cells subsequently reside in a position and express markers associated with highly quiescent HBCs. Taken together, these observations further provide the basis for a new model to explain the role of Mash1-labeled cell fates between the embryonically-labeled OPPs and postnatally-labeled GBCs, and imply that these are distinct cell types.
Figure 3.1. Effective Labeling of Mash1+ Cells in Mouse OE

(A) Schematic of the genetic construct utilized to irreversibly label Mash1-expressing cells. CreERT2 is expressed from the endogenous Mash1 locus. Induction with tamoxifen leads to nuclear translocation of CreERT2, which can activate transcription of reporter protein message by splicing out the preceding “stop” sequence. (B) Schematic of the OE demonstrating the selective labeling of a subset of GBCs. This is the cell type targeted for initial labeling in Mash1-CreERT2; Rosa-tdtomato mice after tamoxifen induction. (C) Representative OE section of Mash1-CreER; Rosa-tdtomato mouse 3 days after tamoxifen induction. The majority of tdtomato-labeled cells are found within the basal compartment, below the GAP43-immunostained immature neurons. (D) Reporter-labeled cells reside predominantly in the neuronal layer by 3 weeks after labeling. Representative image of OE section demonstrating reporter-labeled cells residing above the GAP43-immunostained immature neuronal layer. A dendritic process is clearly visible protruding from one of the labeled cells. (E) Quantification of cells found within the basal zone (within one cell diameter of the basal lamina) or neuronal layer (all cells found above the basal layer but below the sustentacular layer). 3 days after tamoxifen induction, the labeled cells are predominantly found in the basal compartment (n=2, mean +/- standard deviation). (F) Quantification of basal and apical position of cells 3 weeks after labeling. Labeled cells have migrated from the basal compartment to the neuronal compartment (n=2, mean +/- standard deviation). (G-H) A subset of reporter-labeled cells express O/E1 3 days after labeling. High power images of two confocal planes through the same area of tissue are shown. Tissue is immunostained with anti-O/E1 antibody
(green). Arrows and arrowheads provide visual cues to identify labeled cells visualized in different planes. Scale bars = 10 µm.
Figure 3.2  Reporter-labeled cells persist in the OE

(A-B) Representative hemisections of mice sacrificed 3 days (A) or 2 months (B) after tamoxifen labeling at P7. There is a clear decrease in the number of cells present at 3 months. No clustering of cells is noted in the 3 month chase, indicating that labeled cells do not continue to expand. To improve visualization, images were desaturated and color inverted using Adobe Photoshop software. Scale bars = 50 µm. (C) Density of labeling decreases within weeks of tamoxifen labeling. Mash1-CreER ; Rosa-tdtomato mice labeled at P7 were sacrificed at multiple timepoints through 3 months. The density of reporter + cells counted in OE sections is shown. Though the number of labeled cells decreases over time, a significant number of labeled cells persist through 3 months. (D) The proportion of labeled cells found within the basal layer decreases rapidly then stabilizes. The number of cells residing in the basal (black bars) and neuronal (white bars) layers were added for all mice in each chase category, and are presented as the percentage of total counted cells. (n=2 for each timepoint).
A

B

C

D

Chase Period After Tamoxifen Injection

Chase Period After Tamoxifen Injection

3 Day 10 Day 3 Wk 2 Mo 3 Mo

Percentage of Cells

Baseline
Neuronal

Basal Neuronal

62
Figure 3.3 Olfactory Bulbectomy Depletes the OE of Reporter-Labeled Cells

Reporter-labeled cells are greatly diminished on the lesioned side 30 days after bulbectomy. (A) DAPI-stained coronal section of Mash1-CreER ; Rosa-tdtomato mouse sacrificed 30 days after bulbectomy. Only a few reporter-labeled cells remain in the OE on the lesioned side. High-power views of the unlesioned (C) and lesioned (D) are shown. (B) Comparison of reporter-labeled cell density of two hemisections after olfactory bulbectomy. The number of cells labeled in the OE of the lesioned side (filled diamonds) decreases dramatically by 30 days compared to the unlesioned side (open squares). (5 days n=3, 10 days n=4, 30 days n=2) Scale bars = 200 µm for (A) and 20 µm for (C-D).
Figure 3.4 Repertoire of differentiation in Mash1+ cells is restricted after birth

Representative OE Sections from Mash-CreER ; Rosa-tdtomato ; K17-GFP mice tamoxifen injected at either P7 (A-B) or E12 (C-D) and sacrificed 3 weeks later. Reporter-labeled cells from the P7-injected mice are exclusively round cells in the neuronal layer, many with visible dendritic processes (A-B). Clusters of cells demonstrating a distinct sustentacular morphology are found only in the E12-injected mice (C). HBCs labeled with both K17-GFP and tdtomato are also found exclusively in the E12-injected mice (D). Red = intrinsic tdtomato fluorescence, Green = intrinsic EGFP fluorescence. Scale bars = 20 µm.
A  P7 Injected

B

tdTomato
K17-GFP

C  E12 Injected

D
Figure 3.5 Reporter-labeled HBCs persist after Olfactory Bulbectomy

Coronal section of an E12 tamoxifen-induced Mash1-CreER ; Rosa-tdtomato mouse, bulbectomized at P14 and sacrificed 5 days later. There is a dramatic reduction of cell labeling on the lesioned side (A). High-powered view of the unlesioned side (B) demonstrates labeling of both neurons and HBCs, whereas on the lesioned side, the few cells that remain are predominantly HBCs (C). Scale bars = 400 µm (A) and 20 µm (B-C).
Conclusion

The continual neuroregeneration that takes place in the mammalian OE throughout the life of the animal provides a system with great therapeutic potential. How this tissue maintains this capacity is poorly understood. One hypothesis is that similar mechanisms that are employed during the embryogenic creation of the OE remain active in the adult. The experiments described in this dissertation provide evidence that this may not be the case. First, no role in controlling adult neurogenesis was found for TGFβ signaling, an important embryonic regulator. Second, labeling of Mash1-expressing cells demonstrated that this transcription factor appears to label a distinct population of multipotent progenitors in the embryo not seen in postnatally-labeled mice. These findings have important clinical implications.

Several psychiatric and neurological diseases have been hypothesized to result from defects in brain development. Brain development occurs in various stages, and continues into adulthood. That fundamental differences exist in the regulation of OE neurogenesis between the embryonic and postnatal period highlights the importance of studying neurodevelopment at all its various stages, as mechanisms discovered during one stage may not be generalizable. That alternative regulator pathways may be involved at different stages also suggests that defects in neurodevelopment may occur in the postnatal animal after a normal embryonic development, which broadens the window of therapeutic intervention.

Furthermore, the experiments described provide important insights into the challenges that exist in harnessing the neurogenic potential of the OE. Given its anatomic
accessibility, the OE may provide an ideal location for harvesting neural stem cells for therapeutic applications. Understanding the nature of these stem cells is an important first step in actualizing this goal. It is equally important to identify unique molecular signatures on these stem cells to allow proper isolation and purification. Mash1 is a marker that labels a subset of GBCs that has been utilized for this purpose. These data suggest that Mash1 may not be a suitable candidate for isolating a multipotent and continually-proliferating progenitor from adult OE. Further work is needed to determine whether there exists a subset of GBCs that retain this capacity, and if so, a suitable molecular marker to identify them. However, it is possible that this stem cell capacity is limited to HBCs, and efforts to isolate and study this important stem cell population may be the key to unlocking the therapeutic potential of OE.
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