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The role of *Foxg1* in the development of neural stem cells of the olfactory epithelium

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Abstract

The olfactory epithelium (OE) of the mouse is an excellent model system for studying principles of neural stem cell biology because of its well-defined neuronal lineage and its ability to regenerate throughout life. To approach the molecular mechanisms of stem cell regulation in the OE, we have focused on *Foxg1*, also known as brain factor-1, which is a member of the Forkhead transcription factor family. *Foxg1*$^{-/-}$ mice show major defects in the OE at birth, suggesting that *Foxg1* plays an important role in OE development. We find that *Foxg1* is expressed in cells within the basal compartment of the OE, the location where OE stem and progenitor are known to reside. Since FoxG1 is known to regulate proliferation of neuronal progenitor cells during telencephalon development, we performed BrdU pulse-chase of *Sox2*-expressing neural stem cells during primary OE neurogenesis. We found the percentage of *Sox2*-expressing cells that retained BrdU was twice as high in *Foxg1*$^{-/-}$ OE as in wildtypes, suggesting that these cells are delayed and/or halted in their development in the absence of *Foxg1*. Our findings suggest that the proliferation and/or subsequent differentiation of *Sox2*-expressing neural stem cells in the OE are regulated by *Foxg1*.

Keywords

Mouse; neurogenesis; olfactory epithelium; neuronal progenitor; neural stem cell; proliferation; Forkhead; transcription factor; BrdU; *Mash1*; *Ngn1*; *Sox2*; TGF-β; FGF; olfactory receptor neuron

1. The OE neural stem cell

In the past decade, increasing attention has been paid to address the question of how cell-autonomous and non-autonomous molecular mechanisms interact to control neurogenesis 1-5. Such information is of particular importance for understanding the behavior of stem cells in the context of their use as a potential source of treatment for injured or diseased nervous system tissue.

In order to understand the basic principles that govern the generation and regeneration of neurons in the mammals, we have studied the molecular regulation of neurogenesis in a well-characterized neurogenic epithelium, the olfactory epithelium (OE) of the mouse 1, 3. We use OE as a model system, both because of its capacity for continual neurogenesis 6,

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and because of several of its properties simplify the study of neurogenesis 1-3. The OE consists of one major type of neuron, the olfactory receptor neuron (ORN), as well as the stem and progenitor cells that give rise to it. Together, these cell types comprise the OE neuronal lineage, the stages of which can be clearly defined by molecular markers expressed by cells at different stages of neuronal differentiation. In addition, the OE contains an intrinsic glial cell population, the sustentacular cells, which recent evidence suggests may be derived from the same early stem cells as ORNs 1, 6-10.

Figure 1 shows a schematic cartoon of OE neurogenesis. This process begins with OE neural stem cells, which retain a self-renewing ability. These stem cells give rise to the first transit amplifying progenitors (TA progenitors), which express the *Mammalian Achate Scute Homolog 1* gene (*Mash1*; also known as *Asl1*), a proneural gene that encodes a basic helix-loop-helix (bHLH) transcription factor 11-16. *Mash1*-expressing progenitors in turn give rise to the second TA progenitors, immediate neuronal precursors (INPs), which express a different bHLH proneural gene, *Neurogenin1* (*Ngn1*) 15-17. INPs divide and give rise to daughter cells that undergo terminal differentiation into ORN, which express the neuronal cell adhesion molecule *Ncam* 18, 19, and subsequently mature to express the olfactory marker protein, *Omp* 18, 20.

Each stage of neurogenesis is defined both by the expression of particular marker genes and by a generally consistent histological arrangement of the cells within the OE, which becomes apparent after about day 14 of gestation in the mouse 21-23. Studies suggest that stem and TA progenitors are components of the so-called “globose” basal cell (GBC) population, and reside in the basal compartment of the OE atop the “horizontal” basal cells which are adjacent to the basal lamina 1, 5, 18, 24. Once TA progenitors commit to the ORN lineage and undergo postmitotic differentiation, the immature ORNs start migrating from the basal compartment toward a more apical position in the OE, and finally become the bipolar mature ORNs, extending dendrites to the nasal cavity and axons through the OE to the olfactory bulb (Figure 1).

The OE also provides a useful model for studying principles of stem cell maintenance and regeneration of the nervous system 1-5. The OE retains not only the ability to generate neurons throughout life (it has been estimated that ORN turnover rate is 3-12 months in rodents 25, 26), but also a distinct regenerative ability following injury. Studies have shown that, after surgical or chemical ablation the OE undergoes massive cell division and restores almost complete OE formation within two weeks 6, 11, 27. These characteristics strongly suggest that the OE maintains its neural stem cells not only during embryonic development, but also throughout adult life. Thus, unlike other regions of the nervous system, the OE provides a system in which it is possible to investigate neural stem cell behavior during regeneration in the adult nervous system, as well as during development.

Despite these advantages, the detailed molecular regulation of the expansion and differentiation of OE neural stem cells is not fully understood. To date, *Sox2* is the best molecular marker of the neuronal stem cell of the OE 3, 28. *Sox2* is an SRY transcription factor of the *SoxB1* family, and it is expressed in multipotent stem cells throughout the neural primordia 29-31. It has been shown that cells expressing *Sox2* are capable of both self –renewal and differentiation along different developmental pathways, suggesting that *Sox2* expression identifies a stem cell pool 32. *Sox2* is expressed in the OE as well, and importantly it is detected in the basal layer where stem cells are known to be resided 1, 3.

2. Role of *Foxg1* in OE stem cell development

To investigate the molecular regulation of stem cells in the OE, we have focused on *Foxg1* (also known brain factor-1), a member of the Forkhead or Fox proteins which comprise a

large family of “winged-helix” transcription factors that regulate diverse developmental processes in mammals 33. *Foxg1* has been shown to be important in regulating the development of numerous anterior neural structures, including the cerebral cortex, ventral telencephalon, retina, and OE 34-39.

In the OE, *Foxg1* expression is detected early during development when the olfactory placode is forming 39, 40. In *Foxg1* null embryos, OE formation is initiated; however the OE is greatly reduced in size or absent by birth 39-41. Such findings suggest that *Foxg1* is expressed by early progenitors and/or stem cells of the ORN lineage, and that it plays a role in controlling their expansion. To test this hypothesis, we performed in situ hybridization (ISH) using a *Foxg1* cRNA probe at day 14.5 of gestation (E14.5) in wildtype mice OE (Figure 2). *Foxg1* expression in the OE is restricted to the basal layer, where early progenitors such as *Ngn1*-expressing INPs reside 2, 15, 16, 42.

Several lines of study have demonstrated a decrease in proliferation of neuronal progenitor cells in *Foxg1*$^{-/-}$ mice, and this is thought to contribute to the severe hypoplasia observed in the *Foxg1*$^{-/-}$ telencephalon 37, 43. Thus, we hypothesized that the early failure of OE development in *Foxg1*$^{-/-}$ embryos is due to a reduction of stem cell proliferation and/or successive stages of differentiation, resulting in the loss of OE neuronal cells by birth.

To test this hypothesis, pulse-chase BrdU incorporation experiments were performed. We reasoned that if rapidly-dividing neural stem cells were blocked from dividing or differentiating in the OE of *Foxg1*$^{-/-}$ mice, an increase in the number of BrdU-retaining cells would be observed in *Foxg1*$^{-/-}$ OE, but not in the OE of wildtype littermates. BrdU was injected into pregnant dams at E10, when the olfactory pit has formed and cells at all stages of the lineage can be observed in the invaginating neuroepithelium (primary OE neurogenesis)28. Embryos were collected 52 hours later, and processed for *Sox2* ISH and anti-BrdU immunofluorescence 44. We chose this paradigm because (1) OE neurogenesis initiates at E9-10 17,28; (2) OE neuronal progenitor cell-cycle length is estimated to be less than or equal to 17 hrs (with approximately 8 hours of S phase) in embryonic OE 19; and (3) the OE of *Foxg1*$^{-/-}$ is already noticeably hypoplastic and much thinner than normal by E12.5 39, 42. We quantified how many cells were double positive for *Sox2* ISH and BrdU immunofluorescence in the OE, as shown in Figure 3. When these cells were counted, it was found that the percentage of *Sox2*-expressing cells that retained BrdU in the pulse-chase paradigm was twice as high in *Foxg1*$^{-/-}$ OE as in wildtype OE (Figure 3C). These results indicate that proliferation and/or differentiation of *Sox2*-expressing neural stem cells is reduced in the absence of *Foxg1*. The fact that subsequent neuronal cell stages fail to develop in *Foxg1*$^{-/-}$ 42, further adds to the idea that absence of *Foxg1* results in an early block in OE neurogenesis.

3. Conclusions and future directions

Our laboratory's research focuses on understanding how interacting developmental signaling pathways govern OE neurogenesis. We have shown that multiple factors, including fibroblast growth factors (FGF) and TGF-β superfamily members are involved in regulating OE neurogenesis 16, 19, 28, 45, 46. FGFs are important proneurogenic factors for many cells of neuroectodermal origin. Among the FGFs, we have shown *Fgf8* to be expressed and involved in the maintenance of the OE neural stem cell population 28. *Fgf8* appears to exert its major neurogenic effect early in development, during the initial invagination of the olfactory pit and the establishment of the neuronal lineage during primary OE neurogenesis. In contrast, our studies indicate that TGF-βs are negative regulators of neurogenesis in the OE and other sensory epithelia, and that TGF-βs mediate feedback inhibition of stem and progenitor cell proliferation and/or fate choice in these tissues 16, 44, 47 . One member of

the TGF-β superfamily, GDF11, is made by ORNs and INPs within the OE proper. GDF11 has the ability to inhibit OE neurogenesis by reversibly arresting the cell divisions of INPs, an effect that is accompanied by increased expression of the cyclin-dependent kinase inhibitor (CKI), p27Kip1. Moreover, *Gdf11* null mice show an increase in OE neurogenesis in vivo, with increased numbers of proliferating progenitors (INPs), as well as increased OE thickness and increased numbers of ORNs 16, 44.

Interestingly, FoxG1 has been shown to be involved in modulating both FGF and TGF-β signaling pathways. FoxG1 can be phosphorylated by FGF signaling, which promotes the nuclear exportation of FoxG1 and consequently promotes neuronal differentiation 48. FoxG1 has also been shown to interact with Smad-containing transcriptional complexes, which mediate canonical TGF-β signaling, and this can regulate the expression of downstream target genes such as the CKI, *p21Cip1* 49-51. From these studies, we hypothesize that stem and progenitor cell-specific intrinsic factors control and modulate extrinsic signals, to direct these cells in their choice of fates within their developmental niche. Currently, we are investigating the possibility that FoxG1 modulates these signaling pathways in OE development.

Materials and Methods

Animals

Foxg1$^{cre/cre}$ mice, in which the *Foxg1* coding sequence is replaced by *Cre* 36, were obtained by intercrossing *Foxg1*$^{+/cre}$ mice maintained on a Swiss Webster (Harlan) background. Because *Foxg1*$^{cre/cre}$ mice have been used previously for studying *Foxg1*-null phenotypes, and show a phenotype identical to another *Foxg1* null allele, *Foxg1*$^{lacz/lacz}$ 37, 52, *Foxg1*$^{cre/cre}$ mice are designated *Foxg1*$^{-/-}$ in the text of this paper. Midday of the day of vaginal plug detection was designated embryonic day 0.5 (E0.5). All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

In Situ Hybridization (ISH)

Dissected tissues were fixed in 4 % paraformaldehyde (PFA)/phosphate buffered saline (PBS), cryoprotected in 30 % sucrose/PBS, and embedded as described 15. Embedded tissue was sectioned on a cryostat at 12-20 μm, depending on the particular analysis. ISH using digoxigenin-labeled cRNA probes was performed according to published protocols 2. Probes used in this study were generated from: 2.0 kb of mouse *Ngn1* genomic DNA fragment containing ORF 53, 0.75 kb mouse *Sox2* partial cDNA (1281-2029 bp of Genbank X94127) 3; 1.3 kb mouse *Foxg1* cDNA (1391-2688 bp of Genbank NM_008241, gifted from Dr. E. Lai).

Detection of BrdU incorporation

For 52 hr BrdU pulse-chase experiment, pregnant mice were given two injections of BrdU (BrdU, Sigma, 50μg/gm body weight; 1 hour interval) at E10.0, and euthanized 52 hours later. Dissected embryo heads were fixed in 4 % PFA/PBS and cryoprotected in 30 % Sucrose/PBS. Embedded heads were sectioned on a cryostat at 12 μm. Cryosections were processed for anti-BrdU immunoreactivity according to published methods 15. For the double staining, *Sox2* ISH was performed prior to BrdU immunostaining. BrdU^{+} cells were labeled with monoclonal rat anti-BrdU (clone BU1/75-ICR1; 1:1000 dilution of ascites fluid, Harlan), and detected with Texas Red-conjugated goat anti-rat IgG (1:100, Jackson ImmunoResearch). For intensity quantification, the pixel fluorescent intensity sum of individual cells was measured using Axio Vision software (Zeiss). The cells exhibiting more than 50% of the highest intensity of the field were scored.

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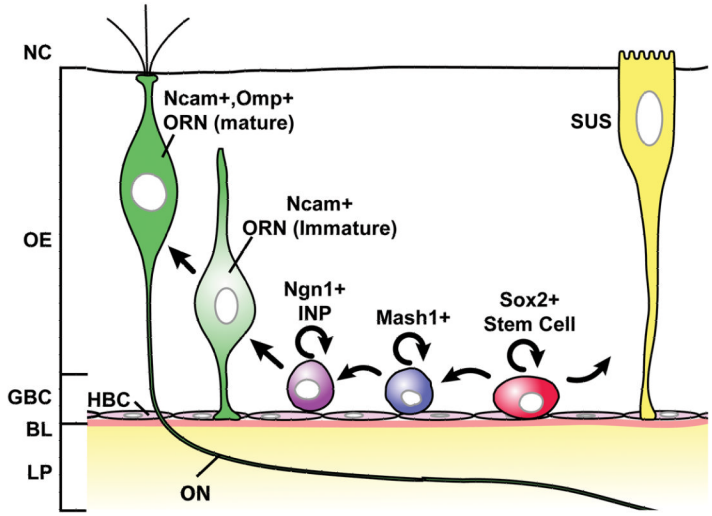

Figure 1.

Scheme of the neuronal differentiation pathway and histological arrangement of cells in the OE. Self-renewing neuronal stem cells (red) give rise to transit amplifying progenitors that express *Mash1* (blue) followed by immediate neuronal precursors (INP) that express *Ngn1* (purple). These cells are detected in the globose basal cell (GBC) layer. INPs divide and daughter cells differentiate into olfactory receptor neurons (ORN, green), which are distinguished by *Ncam* expression. ORNs subsequently mature to express *Omp*. OE stem cells also give rise to sustentacular cells (SUS, yellow; glial lineage). BL, basal lamina; HBC, horizontal basal cells; LP, lamina propria; NC, nasal cavity; OE, olfactory epithelium; ON, olfactory nerve (axons of ORNs).

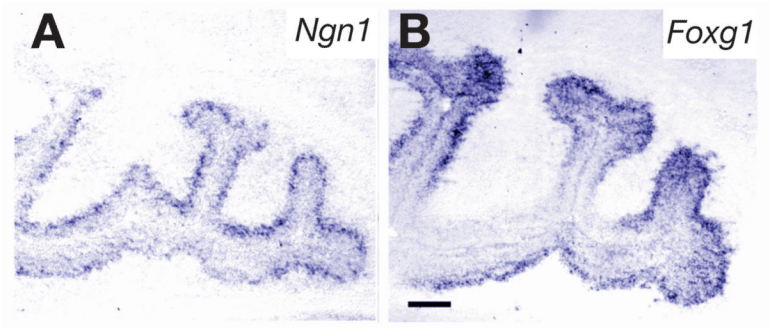

Figure 2.
Expression of *Ngn1* and *Foxg1* in the OE at E14.5. ISH with (A) *Ngn1* probe and (B) *Foxg1* probe. *Ngn1* expression is detected in the basal compartment of the OE where the stem and progenitor cells reside. *Foxg1* expression is detected in the basal layer, similar to the *Ngn1* expression pattern. Scale bar = 100μm. (copyright 2002, Wiley, Hoboken, NJ, USA)

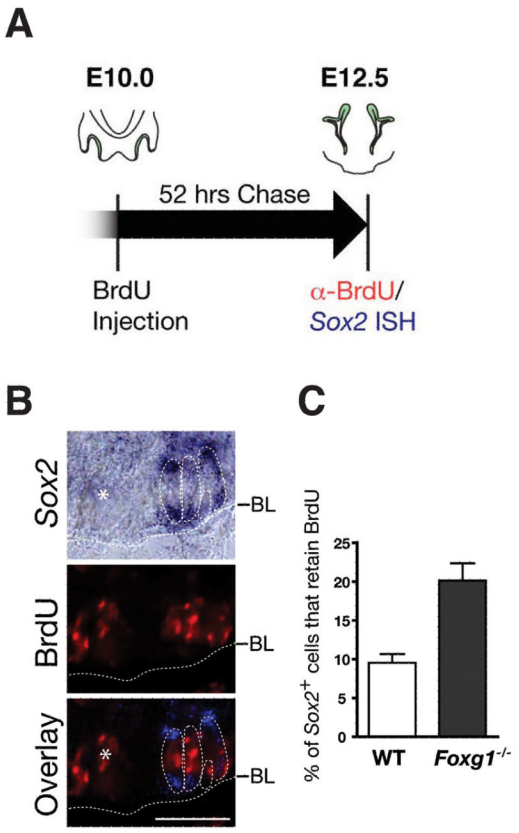

Figure 3.

BrdU 52hr pulse-chase experiment. (A) Scheme of analysis for BrdU pulse-chase experiment. BrdU is injected twice at a 1hr interval at E10.0, embryos are dissected 52 hrs later, and *Sox2* ISH and BrdU immunostaining is performed. (B) *Sox2* ISH and BrdU immuno double staining at E12.5. Images show representative staining. Overlay indicates BrdU image superimposed with digitally converted *Sox2* ISH image. Asterisk indicates BrdU positive, *Sox2* negative cells; *Sox2*/BrdU double positive cells are circled with white break line. BL, basal lamina. Scale bar: 10 μm. (C) A greater percentage of *Sox2* positive cells retaining high levels of BrdU were observed in *Foxg1*$^{-/-}$ animals when compared to wildtype littermates. Over 50% of fluorescent intensity for BrdU immuno reactivity between background and highest intensity points in the field were scored. Quantification results are illustrated as a histogram of mean ± SEM (n=3 animals of each genotype).