Review

Identification and molecular regulation of neural stem cells in the olfactory epithelium

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Abstract

The sensory neurons that subserve olfaction, olfactory receptor neurons (ORNs), are regenerated throughout life, making the neuroepithelium in which they reside [the olfactory epithelium (OE)] an excellent model for studying how intrinsic and extrinsic factors regulate stem cell dynamics and neurogenesis during development and regeneration. Numerous studies indicate that transcription factors and signaling molecules together regulate generation of ORNs from stem and progenitor cells during development, and work on regenerative neurogenesis indicates that these same factors may operate at postnatal ages as well. This review describes our current knowledge of the identity of the OE neural stem cell; the different cell types that are thought to be the progeny (directly or indirectly) of this stem cell; and the factors that influence cell differentiation in the OE neuronal lineage. We review data suggesting that (1) the ORN lineage contains three distinct proliferating cell types—a stem cell and two populations of transit amplifying cells; (2) in established OE, these three cell types are present within the basal cell compartment of the epithelium; and (3) the stem cell that gives rise ultimately to ORNs may also generate two glial cell types of the primary olfactory pathway: sustentacular cells (SUS), which lie within OE proper; and olfactory ensheathing cells (OEC), which envelope the olfactory nerve. In addition, we describe factors that are both made by and found within the microenvironment of OE stem and progenitor cells, and which exert crucial growth regulatory effects on these cells. Thus, as with other regenerating tissues, the basis of regeneration in the OE appears be a population of stem cells, which resides within a microenvironment (niche) consisting of factors crucial for maintenance of its capacity for proliferation and differentiation.

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Introduction

The olfactory epithelium (OE) of the mouse provides an effective model for studying principles of stem cell renewal and differentiation. Unlike other neural regions, neurogenesis proceeds throughout life in OE, allowing studies of stem cell behavior to be conducted in the regenerating adult nervous system, as well as during development [1]. Studies of mouse OE have shown that olfactory receptor neurons (ORNs) are products of a lineage that contains distinct proliferating cell types (Fig. 1A): (1) stem cells, which give rise to (2) transit amplifying progenitors that express Mammalian Achaete Scute Homolog 1 (Mash1), a proneural gene; Mash1 positive (+) cells give rise to (3) a second transit amplifying progenitor, the immediate neuronal precursor (INP), which is distinguished by expression of the proneural gene Neurogenin1 (Ngn1). The INP divides to give rise to daughter cells that differentiate into ORNs [2]. The OE also contains supporting or sustentacular (SUS) cells, analogous to glial cells of the brain [3]. Each of these cell types occupies a different position within the neuroepithelium: the stem cell and transit-amplifying cells are found in the basal compartment, close to the basal lamina of the epithelium, while SUS cells form the apical layer of the OE, adjacent to the nasal cavity (Fig. 1A). ORNs are situated in an intermediate zone between these basal and apical layers, and make up the bulk of the OE. Structurally, the olfactory neuroepithelium resembles the germinative neuroepithelia of the embryo that gives rise to the central nervous system (CNS) [4], but is much simpler in that it produces only one type of neuron. This characteristic makes it possible to study the molecular regulation of neurogenesis in OE, but also has facilitated the identification of cells at different stages in the ORN lineage, as well as the cell and molecular interactions needed to generate ORNs and maintain them at an appropriate number [5]. Below we describe current views on the identity and function of the neural stem cell of the OE, as well what is known concerning molecular regulation of the various cell types it is thought to generate.

The identity of the neural stem cell in the OE

Globose basal cells versus horizontal basal cells

Newly-generated neurons in the OE of adult rodents are the result of mitoses that occur in the basal compartment of the epithelium, apparently in two phases, a slow phase and a rapid phase. Stem cells are thought to undergo slow, asymmetric cell divisions, which result in both maintenance of the stem cell population and generation of a pool of more rapidly-dividing transit amplifying progenitors, committed to a neuronal fate [6]. Evidence from both developmental and regeneration studies indicate that there are two distinct populations of transit amplifying progenitors in OE, one of which is a daughter of the stem cell and expresses the proneural gene, Mash1; the second, which expresses the proneural gene Neurogenin1 (Ngn1), is the daughter of Mash1-expressing progenitors, and can also undergo one or two rounds of symmetric cell divisions [2,7,8]. The progeny of this second transit-amplifying cell, sometimes referred to as the intermediate neuronal precursor (INP; [9]), undergo terminal differentiation into ORNs, which can be distinguished by various markers, such as the neural cell adhesion molecule NCAM, neuronspecific tubulin, and olfactory marker protein [2,10,11].

All three mitotic cell populations remain in the basal compartment of the OE throughout postnatal life. Since this compartment contains two morphologically distinct cell types, horizontal basal cells (HBCs) and globose basal cells (GBCs) (Fig. 1A), there has been difficulty regarding the identification of the stem cell population on the basis of location and mitotic activity alone, and this has prompted the search for unique molecular markers for the stem cell [12–14].

Some tissue culture experiments have provided evidence suggesting that a subpopulation of HBCs reside in a microenvironment reminiscent of other stem cell niches; this combined with a retrospective analysis of their proliferative capacity, has been used to argue that these HBCs represent a stem cell population [13]. In particular, the HBC stem cell candidates, which express keratin intermediate filaments [9], also express intercellular adhesion molecule-1 (ICAM1) and a number of integrins [13]. However, expression of these proteins, as well as epidermal growth factor receptor (EGF-R) is not limited to HBCs: OECs, GBCs, and SUS cells express some of the same integrins [13,15]; and SUS cells and cells of the lamina propria, EGF-R [13]. The fact that HBCs are responsive to EGF, a factor shown to be mitogenic for stem cells in the CNS (e.g., [16]), is interesting. However, HBCs do not seem to express any neuronal progenitor markers [2] or known multipotential stem cell markers (C.L. Beites and A.L. Calof, unpublished observations). HBCs also appear to be a relatively quiescent population, like label-retaining cells in other stem cell systems [13,17], but this may reflect a high level of differentiation rather than stem cell properties.

Alternatively, it may be the case that HBCs are analogous to the ependymal cells that line the ventricles of the postnatal...
mammalian brain, also relatively quiescent in terms of their divisions and once thought to be neural stem cells [18,19]. Indeed, although morphologically very different, ependymal cells and HBCs are similar in several respects: both cell types divide rarely, are capable of being propagated and generating ‘‘neurospheres’’ in culture, and are located in close apposition to neuronal progenitor cells [18].

Given the fact that the position of HBCs within the OE is comparable to the location of ependymal cells within the subventricular zone (SVZ), a source of neural stem cells in the postnatal brain; along with the observation that ependymal cells contribute to the SVZ stem cell niche by secreting important regulators of neurogenesis [20]; it may be that HBCs, rather than being neural stem cells, are rather an important contributor to the stem cell niche, creating a neurogenic environment for the adjacent suprabasal layer where true stem cells reside.

The population in this adjacent layer (GBCs) differ from HBCs in that they are round in shape, cytokeratin-negative, and are known to contain the two populations of transit amplifying progenitors in the ORN lineage: Mash1-expressing progenitors and Ngn1-expressing INPs [7,8,21]. Several lines of evidence suggest that the neural stem cells of the OE lie among the GBCs: Data from retroviral lineage analyses, including some from models of injury-induced OE regeneration, support the idea that a subpopulation of GBCs are multipotent progenitors [12,22]. In vitro experiments also indicate the presence of neuronal stem cells in a small subpopulation of GBCs, and in addition demonstrate that these cells are responsive to FGF2 [8,14,23]; HBCs, in contrast, do not seem to be responsive to FGF2 [13].

Overall, the bulk of data appear to support the idea that OE neural stem cells, at least those present in the established epithelium in rodents, are a subpopulation of GBCs.

Interestingly, despite the controversy that exists concerning the morphological identity of rodent OE stem cells, in human OE there is no morphological distinction between HBCs and GBCs [24]. Both the cells in the layer immediately adjacent to the basal lamina, and those cells directly above them, are “globose” cells, resembling rodent GBCs. Furthermore, cells in both layers express cytokeratins, normally only found in rodent HBCs. Thus, cells in the basal compartment of human OE seem to possess characteristics of both GBCs and HBCs in rodents. This difference in human OE underlies the significance of studies involving both HBCs and GBCs. It will be important to further characterize both types of rodent basal cells, to understand their functional role in development and regeneration, and to decipher the similarities and differences between these cell types in rodents and man. Ultimately, such comparisons will help us to understand whether all cell types in the OE are generated by a single multipotent stem cell, and whether the information gained from studies of rodent species will aid in our understanding of neuronal regeneration in man.

Does the OE neural stem cell give rise to both neuronal and glial cells?

Sustentacular cell origins

Numerous studies indicate that the ORNs’ differentiation pathway consists of specific populations of proliferating
progenitor cells, derived ultimately from a stem cell, which become progressively more restricted in their developmental potential as they give rise to terminally-differentiated (post-mitotic) ORNs. The data taken from studies, from our lab and others, strongly support the lineage diagrammed in Fig. 1A: According to this view, stem cells generate Mash1-expressing neuronal progenitors, which in turn generate Ngn1-expressing INPs; INPs are committed to generating daughter cells that exit the cell cycle and become differentiated ORNs (reviewed in [2]). In contrast, the developmental origins of SUS cells, as well as their significance in the OE neurogenic pathway, are not yet completely understood.

SUS cells lie in a single layer on the apical surface of the OE, and possess thin cytoplasmic projections that terminate as endfeet at the basal lamina [3]. This self-renewing, non-neuronal cell type expresses enzymes involved in metabolizing foreign compounds (cytochrome p450, glutathione-S-transferase mu 2, and carbonyl reductase 2; [25]), suggesting a role for SUS cells in detoxifying many of the noxious substances present in the air, to which the OE is exposed. SUS cells also phagocytose dead ORNs [26], and much like the glia of the brain, probably provide structural support to ORNs.

Since SUS cells appear to have some of the characteristics of glial cells, the question as to whether they share a common lineage with ORNs, as appears to be the case for many neuronal cell types of the brain, still remains [27]. Antibody markers have provided some information in this regard. Some monoclonal antibodies, such as SUS1 and SUS4 [28,29], label the SUS cell layer, but not cells beneath this layer in the OE, which would appear to suggest that the ORN and SUS lineages are separate (cf. [9]). However, antibodies to the intermediate filament protein nestin, thought to be a neural stem cell marker in some regions of the CNS, appears to label the endfeet of SUS cells, albeit only in adult tissues ([30,31]; S. Kawauchi and A.L. Calof, unpublished observations). However, both SUS1 and nestin antibodies also label cells of Bowman’s glands, which lie within the lamina propria of the OE, suggesting that Bowman’s gland epithelial cells and SUS cells may share a common progenitor, as has been hypothesized previously [9,22]. Other reports also indicate that SUS cells and respiratory epithelium share some antigenic properties [25], suggesting that SUS cells may not be part of the OE neural lineage.

However, several lines of evidence support the notion that SUS are lineally related to GBCs and neurons. Experiments in which rodents are subjected to methyl bromide lesioning of OE (a paradigm in which SUS cells, ORNs, and basal cells are destroyed) have shown that antigens normally only expressed on GBCs (e.g., GBC1) are now expressed on cells that have morphological characteristics of HBCs, SUS, and/or ORNs [28]. Such findings suggest that SUS cells and cells of the OE neuronal lineage share common molecular markers, at least under some circumstances. More recently, genetic and transplantation experiments have provided evidence that SUS cells and ORNs may share a common, bipotential progenitor: Murray and colleagues [32] performed an analysis of developmental neurogenesis in Mash1−/− mice, in which INPs and ORNs fail to develop due to genetic disruption of the ORN developmental pathway [33]. The OE of these animals, despite having no differentiated INPs or ORNs, is almost completely comprised of proliferating cells that express molecular markers of both committed neuronal progenitors (Mash1 3′ UTR) and SUS cells (Steel) [32]. These data suggest that, at least during development, the OE contains bipotential progenitors — possibly the stem cells of the OE — which subsequently become restricted to either a neuronal (ORN) or glial (SUS) fate, and that proneural gene (in this case, Mash1) function is required for commitment to the ORN differentiation pathway. This is similar to observations made of the inner ear, another placode-derived sensory structure [34]. Since Mash1-expressing cells are found among the GBC population (Fig. 1A and [7]), these findings also imply that this putative stem cell lies among the GBCs.

This idea is supported by recent experiments in which purified GBCs, transplanted into the OE of methyl bromide-lesioned host mice, appear to give rise to both ORNs and SUS cells [35].

The finding that putative stem cells are greatly expanded in number has led to the development of a screen for OE stem cell markers, based on expansion of their expression in Mash1−/− OE compared to wildtypes. Among the genes whose expression is expanded in this paradigm are Sox2, a neuroepithelial marker in a number of neural regions, and Raldh3, which encodes a retinadehyde dehydrogenase that is rate-limiting in the cellular synthesis of retinoic acid (RA) (Fig. 1C; [5]). Expression of Raldh3 by putative OE neural stem cells is likely to be of particular significance, since RA is known to have widespread functions in vertebrate development: For example, animals in which RA signaling has been depleted show a spectrum of craniofacial malformations that include loss of OE and olfactory bulb tissue [36]. Among the four Raldh family members, only Raldh3 is expressed in the olfactory pit from an early stage [5], implying that RA signaling, mediated by RALDH3, plays an important role in OE development. Indeed, mice null for Raldh3 show aberrant development of the nasolacrimal ducts and morphological defects of the nasal cavity [37], supporting this idea.

Sox2, which encodes an HMG box transcription factor expressed in multipotent stem cells throughout the neural primordium, also displays expanded expression in Mash1−/− OE (Fig. 1B; [5]). In other systems, cells expressing Sox2 are capable of both self-renewal and differentiation along different developmental pathways, suggesting that Sox2 expression identifies a stem cell pool [38]. In normal OE at e14.5 (Fig. 1B), Sox2 is expressed in both basal and apical layers, consistent with the location of mitotically active cells at this early stage of development [4]. However, in Mash1−/− OE,


**Sox2** expression is no longer restricted to apical and basal layers: instead, expression expands to encompass most of the cells in the epithelium, comparable to expression of **Raldh3** and **Mash1** 3’ **UTR** (Fig. 1B; [5]). Altogether, the findings described above lead us to our current view that the **SUS** cell and the ORN are lineally related, arising from the same bipotential neural stem cell in the OE.

**Olfactory ensheathing cell (OEC) origins**

A glial cell type of importance to OE function and development but whose lineal origins remain controversial is the OEC. OECs encircle bundles of ORN axons, are heterogeneous in character, and possess characteristics of both Schwann cells and astrocytes; moreover, they express a variety of cellular markers depending on their location in the olfactory pathway [39]. Transplanted OECs appear to promote recovery in a variety of nerve lesion models [40], and it has been proposed that their presence is the reason that the olfactory system is permissive to axon regrowth [41]. From a clinical perspective, identification of the stem cell that gives rise to OECs may therefore prove of great value, enabling large numbers of OECs to be propagated for use in surgical repair of nerve lesions (e.g., in spinal cord injury).

Surprisingly little experimentation has been devoted to defining the origins of OECs, although evidence exists to suggest that, in chick, they originate from the olfactory placode [42]. The idea that the OE stem cell could be the source of cells destined to reside outside of the epithelium has also been suggested by the presence of cell clusters present around the developing olfactory nerve in the lamina propria, at the time when ORN axon outgrowth is initiated [42,43]. Genetic evidence that the OEC stem cell may reside in OE comes from the study of **distal-less-5** (**Dlx5**) mutant mice. In the absence of **Dlx5** function, olfactory placode development is impaired, leading to the formation of only a rudimentary OE, and no OECs appear to be present in the olfactory bulb nerve layer [44].

Indirect evidence for an OEC stem cell within the OE comes from tissue culture studies by various groups. Work by Au and colleagues has shown that GFAP-expressing ensheathing cells can be generated in vitro from explants of purified embryonic OE [45]. Studies of cell lines created by use of retroviruses to transduce oncogenes into purified ORN progenitors (the entire GBC population) showed that the majority of cell lines that could be immortalized had morphological characteristics of OECs and expressed OEC markers such as GFAP and S100-β [46]. Furthermore, colony-forming assays of purified ORN progenitors (essentially all GBCs), grown at clonal density, yield a low percentage of colonies containing cells that have an OEC-like morphology [23]. Others have reported the presence of astrocyte-like and Schwann-like cells in cultures of olfactory mucosa [10].

Thus, studies of OE growth and development in vivo and in vitro suggest that OECs may originate within OE proper, and thus may be products of a multipotential OE stem cell. However, no data are available to indicate the molecular characteristics of the cells that give rise to OECs. Moreover, the heterogeneity of OEC marker profiles suggests that a dual origin (OE plus another tissue, such as neural crest) of OECs is certainly plausible. If OECs are derived strictly from OE proper, then they would be the only glial cell type that is placode-derived rather than neural crest-derived [47]. Thus, since no definitive lineage tracing study has been performed to demonstrate unequivocally that OECs arise from the olfactory placode or OE proper, their origin remains uncertain at present.

**Molecular control of neurogenesis in the OE**

**Two phases of neurogenesis in OE development**

Several lines of evidence suggest that the molecular signals that result in determination of the olfactory placode and initial establishment of the OE neural lineage (primary neurogenesis) are different from those that regulate ongoing and regenerative neurogenesis (established neurogenesis). Generation of ORNs appears to be influenced by different families of signaling molecules, of which specific members are expressed at distinct developmental ages. In particular, fibroblast growth factor (FGF) superfamily members are important proneurogenic factors, not only for cells of the OE, but also for many cells of neuroectodermal origin [48]. **Fgf8** appears to exert its major neurogenic effect early during development, during initial invagination of the olfactory pit and establishment of the neuronal lineage during primary OE neurogenesis [5,49]. During this time, **Fgf8** expression is highest at the rim of the invaginating pit, coincident with vigorous cell proliferation in this area [5,49]. Interestingly, expression of **Fgf8** decreases as development proceeds. **Fgf2**, conversely, does not appear to be expressed in developing OE, but is highly expressed in mature OE of adult animals [5]. Since both these FGFs have positive actions on stem cells of the OE in vitro [8,14,49], this latter observation suggest that expression of **Fgf2** is an important factor maintaining the stem cell niche in adult OE.

Around e13.5–e14.5, the OE becomes organized into its mature pattern and established neurogenesis is initiated: During this time, SUS cell nuclei become organized as a single apical layer [4] where they begin to self-renew [3], and the overall number of mitotic figures decreases and becomes localized primarily to the basal compartment of the OE [4]. Interestingly, presumptive stem cell markers such as Sox2 and Raldh3 are detected throughout the OE up to about this transition point, but by e14.5, their expression becomes more polarized, to apical and basal layers of the OE (Fig. 1B; [5]; C.L. Beites, S. Kawauchi, and A.L. Calof, unpublished observations). We know that transforming
growth factor-β (TGF-β) superfamily signaling molecules, in particular bone morphogenetic proteins (BMPs) and growth and differentiation factor 11 (GDF11), are important negative regulators of established neurogenesis in the OE, and their expression during development reflects this [21,50,51]: For example, expression of Gdf11, which encodes a secreted autoregulatory factor (GDF11) that mediates feedback inhibition of OE neurogenesis [21], is not detected prior to e12.5 (H.-H. Wu, J. Kim, and A.L. Calof, unpublished observations).

One possibility suggested by these observations is that the role of factors that predominate during primary olfactory neurogenesis (prior to e13.5) is to increase the size of the stem cell pool (e.g., an expansion phase; cf. [52]). Once the OE lineage and pattern of neurogenesis are established, stem cells – which have come to be located in the basal compartment of the OE – may be active primarily in generating later cell types, i.e., committed neuronal progenitors (which give rise to ORNs) and SUS cells. In established OE, newly-produced ORNs in turn will start to regulate total neuron number tightly, through production of the negative regulator GDF11, which inhibits INP proliferation [21]. Thus, our observations suggest that OE development proceeds in a manner analogous to that of the CNS, with stem cell expansion followed by neurogenic and gliogenic phases [52].

**The OE stem cell niche**

One difference between OE and most of the CNS, however, is that regeneration of ORNs occurs throughout life. Thus, the environment of stem cells (their “niche”) must continue to be permissive for stem cell renewal and ORN production. The OE stem cell niche is undoubtedly complex, and remains to be fully defined. Secreted factors that regulate proliferation and differentiation of stem and progenitor cells in the microenvironment of the OE stem cell have been identified. This is illustrated in Fig. 2.

Data concerning the proneurogenic actions of FGFs and their expression in the OE microenvironment have been described above. In addition, several reports indicate that EGF, its receptor, and at least one cognate ligand (TGF-α) are both expressed in the OE microenvironment and stimulate proliferation of HBCs (above and [53–55]). Thus, several factors that stimulate proliferation and/or survival of OE stem and progenitor cells are present in the stem cell niche.

A more puzzling issue, however, is the question of how the antineurogenic actions of the many TGF-βs expressed in the OE and its lamina propria are regulated to permit stem and progenitor cell proliferation to occur when these are needed for neuronal proliferation and regeneration. GDF11, which is expressed by ORNs, acts on INPs to induce reversible cell-cycle arrest [21]. This action may permit INPs to be held in stasis until they are needed to replace dead or dying ORNs in mature OE. Indeed, the observation that INPs are rapidly induced to divide following induction of ORN apoptosis (by olfactory bulbectomy) supports the idea that ORN death removes a feedback inhibitory signal that normally acts to hold progenitor cell proliferation in check [7,56]. However, Gdf11 is also expressed during development and early postnatal ages, when the OE is

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**Fig. 2.** A model of the OE neural stem cell and its derivatives, plus the extrinsic factors that regulate stem and progenitor cells within the OE microenvironment. Stem cells self-renew and also give rise to Mash+ progenitors, which will eventually give rise to ORNs, through an INP stage (neuronal fate). OE stem cells may also give rise to SUS and OEC cell lineages (glial fates). HBCs may be an important part of the OE stem cell niche, creating a neurogenic environment for the adjacent basal layer where stem cells reside. Signaling molecules (both proneurogenic and antineurogenic) are found in different regions: GDF11, Fst, FGF2, and BMP4 found throughout OE; FGF8, BMP7, EGF, and TGF-α in the basal compartment; Fst, BMP7, BMP4, BMP2, and noggin in the LP and/or developing nasal bone (NB).
rapidly expanding in overall size [57]. It appears that secreted antagonists of TGF-βs, which are expressed in the OE microenvirnment, are important in allowing developmental neurogenesis to take place. These proteins, which are expressed in many regions of the body, bind to TGF-βs with high affinity and prevent their interaction with cell-surface receptors [58]. Follistatin, a high-affinity antagonist of GDF11, is expressed both within OE and its underlying stroma, and genetic experiments indicate that it plays a critical role in promoting OE neurogenesis in vivo ([21]; H.-H. Wu and A.L. Calof, unpublished observations). The BMP antagonist noggin, which likely plays a role in maintaining the stem cell niche of the SVZ [20], is expressed in developing cartilage and nasal bone ([51]; C. Crocker and A.L. Calof, unpublished observations), where it may inhibit the actions of locally-synthesized BMPs which have antineurogenic actions in OE, such as BMP2, BMP4, and BMP7 [50,51].

Concluding remarks

An important point emerging from studies on the OE neural stem cell is that many of the molecules expressed within the stem cell microenvironment of the OE are also important in the CNS. It will be interesting to determine whether other similarities exist. For example, do stem cells of the OE express any radial glial cell markers, similar to those expressed by stem cells in the ventricular zone? Will cleavage plane orientation decide cell fate in the OE as it does in the ventral telencephalon [59]? If so, do the factors described above affect this fate, depending upon their localization within the OE? Understanding similarities with other neurogenic regions should provide us with insight into a common regulatory network that defines the neural stem cell state, and will provide us with important knowledge for facilitating neurogenesis in regions of the adult nervous system where regeneration is limited or absent.

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