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Adult c-Kit (+) progenitor cells are necessary for maintenance and regeneration of olfactory neurons

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Abstract

The olfactory epithelium houses chemosensory neurons, which transmit odor information from the nose to the brain. In adult mammals, the olfactory epithelium is a uniquely robust neuroproliferative zone, with the ability to replenish its neuronal and non-neuronal populations due to the presence of germinal basal cells. The stem and progenitor cells of these germinal layers, and their regulatory mechanisms, remain incompletely defined. Here we show that progenitor cells expressing c-Kit, a receptor tyrosine kinase marking stem cells in a variety of embryonic tissues, are required for maintenance of the adult neuroepithelium. Mouse genetic fate-mapping analyses show that embryonically, a c-Kit (+) population contributes to olfactory neurogenesis. In adults under conditions of normal turnover, there is relatively sparse c-Kit (+) progenitor cell (ckPC) activity. However, after experimentally induced neuroepithelial injury, ckPCs are activated such that they reconstitute the neuronal population. There are also occasional non-neuronal cells found to arise from ckPCs. Moreover, the selective depletion of the ckPC population, utilizing temporally controlled targeted *diphtheria* toxin A expression, results in failure of neurogenesis after experimental injury. Analysis of this model indicates that most ckPCs reside among the globose basal cell populations and act downstream of horizontal basal cells, which can serve as stem cells. Identification of the requirement for olfactory c-Kit expressing progenitors in olfactory maintenance provides new insight into the mechanisms involved in adult olfactory neurogenesis. Additionally, we define an important and previously unrecognized site of adult c-Kit activity.

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Keywords

neurogenesis; stem cells; growth factor; receptor tyrosine kinase; olfactory epithelium

INTRODUCTION

Certain regions in the adult mammalian nervous system, including the subventricular zone and the dentate gyrus, retain an ability to replenish neurons (Reynolds and Weiss, 1992; Luskin, 1993; Doetsch et al., 1999; Gage, 2000). The most striking capacity for self-renewal in an adult neuroepithelium occurs in the olfactory epithelium (OE) of the nose (Graziadei and Graziadei, 1979; Hinds et al., 1984; Calof and Chikaraishi, 1989; Schwob et al., 1992; Schwob et al., 1995). Here, neurons are vulnerable and must be replaced to maintain function of a sensory system that is vitally important to most mammals. As in other self-renewing tissues, it is the presence of stem and progenitor cells that underlie the regenerative capacity. OE basal layers contain a heterogeneous progenitor population (Goldstein and Schwob, 1996; Goldstein et al., 1998; Chen et al., 2004; Leung et al., 2007; Fletcher et al., 2011), and the niche signals regulating renewal and differentiation remain incompletely understood.

Olfactory mucosal cells include bipolar receptor neurons, sustentacular and microvillar cells, gland cells, and two categories of basal cells. The horizontal basal cell (HBC), a slowly-dividing keratin-positive population, (Holbrook et al., 1995) has been shown by fate-mapping studies to act as a reserve stem cell (Leung et al., 2007). Retroviral lineage studies (Huard et al., 1998), transplantation assays (Goldstein et al., 1998; Chen et al., 2004), and genetic fate mapping (Gokoffski et al., 2011) indicate that the keratin-negative globose basal cell (GBC) population also contains multipotent cells, some of which also act as label-retaining reserve cells (Jang et al., 2014). Other GBCs function as amplifying progenitors and neuronal precursors, expressing the neurogenic basic helix-loop-helix transcription factors (Guillemot et al., 1993; Gordon et al., 1995; Cau et al., 1997; Manglapus et al., 2004). Given the complexity of the cells within this germinal zone, ongoing controversy exists regarding progenitor cell relationships, and their regulatory mechanisms.

Here, we sought to investigate the role of cells expressing the type III receptor tyrosine kinase c-Kit (which we refer to as c-Kit (+) Progenitor Cells, ckPCs) in OE maintenance. C-Kit signaling has critical roles in adult hematopoiesis, and diverse functions in broad tissue systems during development (Keshet et al., 1991; Bernex et al., 1996; Lennartsson and Ronnstrand, 2012). Previous reports have indicated that *c-Kit* and its ligand *Steel* (also known as Stem Cell Factor (SCF) or Kit ligand) are expressed within the embryonic nasal mucosa (Orr-Urtreger et al., 1990; Guillemot et al., 1993; Murray et al., 2003), but no direct evidence for a functional role has been provided, nor has adult expression been investigated. It is of interest that in embryonic OE from mice lacking *Ascl1* (also known as *Mash1*), few neurons are produced while *Steel* expression appears increased (Guillemot et al., 1993; Murray et al., 2003). These findings suggest a feedback mechanism in which c-Kit signaling may regulate neuronal progenitors. Accordingly, we combined a ckPC-specific fate mapping strategy with experimental olfactory injury to define the role of c-Kit expressing cells in

embryonic and adult neurogenesis. In addition, we utilized the cre/loxP system to direct the temporal expression of latent *diphtheria* toxin to adult ckPCs to selectively deplete this population, permitting a direct examination of the requirement for ckPCs in adult olfactory neuroepithelial maintenance.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee of the University of Miami approved all experiments. The *c-Kit^{CreERT2/+}* mouse line was provided by Dr. Dieter Saur, Technical University of Munich (Klein et al., 2013). The construct was inserted as a knock-in, however, the *c-Kit^{CreERT2/+}* mice are haploinsufficient for c-Kit, presumably due to low expression via the internal ribosomal entry site (IRES). We identify no differences in OE histology, nuclear size, or OE reconstitution between these mice or wild type controls, consistent with the absence of olfactory phenotype in spontaneous Kit heterozygous mutants. Cre reporter mice were obtained from Jackson Lab (Bar Harbor, ME). The *Gt(ROSA)26Sor^{tm1Sor}/J* (Stock Number: 003474) line, which we refer to as *R26R^{LacZ}*, produces β -galactosidase (bgal) following Cre activation. The Tg(CAG-DsRed,-EGFP)5Gae/J (Stock Number: 008605) line, which we refer to as GFP reporter, produces GFP following Cre activation. The *Gt(ROSA)26Sortm1Jpmb/J* (Stock Number: 006331) line, referred to as *ROSA26^{eGFP-DTA}*, functions as a conditional cell ablation model. For fate mapping, *c-Kit^{CreERT2/+}* mice were mated with the Cre reporter lines to obtain compound mutants on a mixed *C57Bl/6; 129S6* background. For conditional deletion of c-Kit-expressing cells, *c-Kit^{CreERT2/+}* mice were crossed with conditional *ROSA26^{eGFP-DTA}* mice. For Cre induction, tamoxifen (Sigma, St. Louis, MO) 10–20 mg/ml in peanut oil (Sigma) was given daily 2 mg intraperitoneally at designated times to adults, or 0.2 mg to postnatal mice. Methimazole lesion was induced by treating 4–8 week old mice with a single intraperitoneal injection of methimazole 75 μ g/g body weight (5 mg/ml solution in PBS). For mitotic labeling, mice were treated with a single intraperitoneal injection of 5-bromodeoxyuridine (BrdU, Sigma) 50 mg/kg 2 hours prior to euthanasia.

Tissue processing

Mouse genotypes were confirmed with PCR from tail biopsies using standard protocols, prior to use. Wild type *C57BL6/J* mice were obtained from Charles River (Wilmington, MA). For embryonic experiments, mice were mated and the day after vaginal plug was designated E 0.5. At desired gestation, pregnant mice were euthanized by CO₂ inhalation followed by decapitation and embryos were harvested. Postnatal mice were euthanized by CO₂ inhalation followed by decapitation. Adult mice were euthanized by exsanguination from perfusion with saline followed by fixative under deep ketamine-xylazine anesthesia. Embryos or postnatal heads were fixed by immersion in 4% paraformaldehyde for 1–2 hours, rinsed in PBS and cryoprotected overnight in 30% sucrose in PBS. After 4% paraformaldehyde perfusion, adult nasal tissue was dissected from surrounding muscle and bone, post fixed 1–2 hours, rinsed in PBS and then treated with 30% sucrose/250 mM EDTA in PBS 2–4 days. Specimens were then embedded in O.C.T. compound (VWR,

Radnor, PA) and frozen in liquid nitrogen. Tissue was cryosectioned at 10 μ m and collected on Superfrost Plus slides (VWR) and stored at -20° degrees.

Antibody characterization

Primary antibodies used in these experiments are described in Table 1. The information regarding these reagents is derived from our data as well as from manufacturers' descriptions.

The rabbit anti- β -galactosidase (MP Biomedical, rabbit polyclonal, cat #55976 RRID: AB_10013481) recognizes the native and denatured enzyme near the C-terminus in dot blot assays. The immunohistochemical pattern appears identical to x-gal reaction.

The chicken anti- β -galactosidase (Abcam, chicken polyclonal, cat #ab9361 RRID: AB_307210) was raised against full length purified *Escherichia coli* protein and has been widely characterized. We found the staining pattern to be identical to x-gal reaction, following citrate heat-mediated antigen retrieval.

The anti-BrdU (BD Biosciences, mouse monoclonal, cat#555627, clone 3D4 RRID: AB_395993) reacts against BrdU but not other nucleotides in single-stranded DNA. Reactivity has been verified by flow cytometry controls and immunocytochemistry on fixed KG1A cell line, per manufacturer.

The rabbit anti-c-Kit (Cell Signaling Technology, rabbit monoclonal, cat#3074, RRID: AB_10829442) detects bands at 120 and 145 kD on Western blots, corresponding to c-Kit. It does not react against other receptor tyrosine kinases in NCI-H526 cells by Western. Also, immunocytochemistry of NCI-H526 cells reveals staining that is absent in control Jurkat cells.

The goat anti-c-Kit (Santa Cruz, goat polyclonal (M14), cat#sc-1494, RRID: AB_631032) has been verified to recognize the 120 and 145 kD bands corresponding to c-Kit on Western blots from HeLa cells, and gives cytoplasmic labeling by immunocytochemistry of HeLa cells. In addition, characterization of the labeling of c-Kit expressing interstitial cells of Cajal using this antibody has been well described (Klein et al., 2013).

The anti-CK18 (Abcam, rabbit monoclonal, cat #ab32118, RRID: AB_736394) has been shown to react specifically with only the keratin 18 isoform in human colonic carcinoma. The antibody is a widely established cell type-specific marker in olfactory mucosa for Bowman's gland acinar and duct cells as well as sustentacular cells. We found citrate heat-mediated antigen retrieval to be required for this reagent.

The anti-CK5 (Abcam, rabbit polyclonal, cat#ab24647, RRID: AB_448212) has been shown to react to specific subsets of breast cancer cell lines expressing only the keratin 5 isoform, without cross-reacting to other isoforms (Cardiff et al., 2013). The cell type-specific labeling in the OE matches exactly the pattern of CK5/6 and CK14 labeling that has been well described to correspond to horizontal basal cells (Holbrook et al., 1995).

The anti-GAP43 (Abcam, rabbit monoclonal, cat#ab75810, RRID: AB_1310252) is specific for the C-terminus of human GAP43. It has been extensively used as a marker for immature olfactory sensory neurons.

The anti-OMP (Wako, goat polyclonal, cat#019-22291, RRID: AB_664696) is highly specific for a soluble acid protein expressed in mature olfactory neurons. The OMP immunohistochemical expression pattern was characterized in detail for several species previously (Keller and Margolis, 1975).

The anti-PCNA (Abcam, mouse monoclonal, cat#ab2426, RRID: AB_303062) recognizes a specific band at 29 kD corresponding to PCNA, on Western blots from HeLa cell lysates. This reactivity can be blocked with a synthetic PCNA peptide. In the OE, the labeling pattern with this reagent localizes only to nuclei of proliferative cell layers, and not to post-mitotic neurons.

The anti-Pax6 (Millipore, rabbit polyclonal, cat#AB2237, RRID: AB_1587367) recognizes the 50 kD band corresponding to Pax6 on Western blots from fetal rat or mouse brain lysates. In addition, the pattern of labeling in the OE using this reagent has been described in detail previously (Guo et al., 2010).

The rabbit anti-SCF (Bioss Inc, rabbit polyclonal, cat#bs-0545R, RRID: AB_10855650) has an appropriate cell type-specific cytoplasmic and surface labeling pattern of hematopoietic progenitors in the rat spleen. In addition, immunolocalization pattern has been verified in rat kidney stem cell niches.

The mouse anti-SCF (Santa Cruz, mouse monoclonal (G-3), cat#sc-13126, RRID: AB_628238) recognizes a band close to 45 kD corresponding to SCF protein on Western blots from HeLa cell lysates and from rat brain. In colon tissue sections, this reagent labels glandular cells with a cytoplasmic and extracellular distribution. We also confirmed a 43 kD band on Western blots from mouse bone marrow lysates.

The antibody TuJ-1 (Covance, mouse monoclonal, cat#MMS-435P, RRID: AB_10063408) recognizes neuron-specific class III β tubulin. It does not react to the tubulin found in glia cells, and its reactivity in immature neurons has been well described by our lab and others (Schwob et al., 1995; Goldstein and Schwob, 1996).

Staining and immunohistochemistry

For visualization of β -galactosidase, tissue was reacted with x-gal staining solution using an x-gal kit (Invitrogen) per instructions. Slides or whole mount tissues were incubated overnight at room temperature. Alternatively, reporter was visualized with anti- β -galactosidase immunochemistry. For immunohistochemistry, slides were rinsed in PBS, and if necessary antigen retrieval was performed using sodium citrate steaming technique. A blocking solution of PBS, 10% normal serum (Jackson ImmunoResearch, West Grove, PA), 4% bovine serum albumin, 5% nonfat dry milk and 0.1% triton x-100 was applied for 30–60 minutes, followed by primary antibody, in the same solution for 1 hour at room temperature or overnight at 4 degrees. Slides were PBS rinsed and incubated with either fluorescent-conjugated secondary antibody or biotinylated secondary (Jackson ImmunoResearch) for 45

minutes in same blocking solution. Fluorescent stained slides were then rinsed and coverslipped with Vectashield containing DAPI (Vector Labs, Burlingame, CA). Cryosections from *c-Kit^{CreERT2/+};GFP* reporter mice were viewed for direct eGFP epifluorescence. For immunoperoxidase staining, the Vectastain Elite ABC kit (Vector) was used per instructions, using DAB substrate as the chromogen, and coverslipped using Vectamount medium (Vector).

Western Blotting

For protein isolation, nasal turbinate and septum mucosal tissue was homogenized in ice-cold RIPA buffer containing protease inhibitor cocktail (Sigma). Following a 30 minute incubation on ice, vortexing every 10 minutes, the cell lysates were centrifuged at 14,000 x g for 15 minutes, and the supernatants were collected and stored at -20°C. Total protein concentration in each sample was quantified by Bradford assay to ensure equal protein loading of 50 mg per lane. Samples were diluted in Laemmli buffer and heated to 100 °C for 5 minutes, analyzed by 10% Bis-Tris polyacrylamide gel electrophoresis, and transferred to PVDF membranes (BioRad, Hercules, CA). Membranes were blocked in 5% non-fat dried milk in Tris buffered saline (TBS) with 0.1% Tween-20. Immunoblot analysis was performed using mouse antibodies against SCF, control rabbit polyclonal antibody against GAPDH (1:1000; Santa Cruz Biotechnology), and HRP-conjugated secondaries (BioRad). Antibodies were diluted in 5% BSA in TBS with 0.1% Tween-20. Membranes were visualized by chemiluminescence (Pierce SuperSignal) on a ChemiDoc imager (BioRad).

Imaging and cell counting

Tissue sections were analyzed on a Nikon epifluorescent microscope or a Zeiss LSM-710 confocal microscope. Images were acquired and analyzed in Image J software. Brightness and contrast were adjusted as needed. In red/green double-labeled images, Adobe Photoshop was used to convert colors to magenta/green. For counting of TuJ-1 or OMP labeled cells, images were opened in ImageJ and epithelial length at the basal lamina was measured along similar anterior-posterior regions of turbinates I, IIa and IIb, for consistency. At least 3 separate sections per animal were analyzed. DAPI labeled nuclei above the basal lamina surrounded by immunostained soma were counted as positive and totaled per 0.5 mm length of epithelium. Data are presented as mean $\pm$ s.d. X-gal stained cells per 0.5 mm epithelial length were counted in similar fashion from light microscopy images. Abercrombie correction was not deemed necessary, as no differences in nuclear size from *c-Kit^{CreERT2/+}* or *c-Kit^{CreERT2/-}* mice were identifiable: mean diameters were 4.00 ± 0.38 versus 4.01 ± 0.29 μ m, $p=0.94$, t-test.

Statistics

Student's t-test or ANOVA followed by Tukey's Multiple Comparison Test was applied for the bgal labeled or neuronal cell counts, including the Tuj-1 or OMP labeled cell quantification.

RESULTS

Selective genetic marking of c-Kit-expressing lineage in olfactory tissue

To determine the role of ckPCs in olfactory neurogenesis, we utilized cre/loxP genetic fate mapping under temporal control of tamoxifen administration (Fig 1A, B). A CreERT2 allele as a knock-in at the endogenous *c-Kit* locus (*c-Kit*^{CreERT2/+} mouse line) was previously generated and validated for the analysis of enteric interstitial cells of Cajal expressing the c-Kit receptor. In the *c-Kit*^{CreERT2/+} mouse line, Cre can be activated in the c-Kit lineage by tamoxifen administration at desired time points during embryogenesis or in adults. By crossing the *c-Kit*^{CreERT2/+} line with Cre reporters, we obtained mice in which either enhanced GFP (GFP) (Fig 1A, C, E) or bgal (Fig 1B, F–H) can be visualized in progeny of c-Kit (+) cells after tamoxifen induction.

During development, differentiated mouse olfactory neurons are identifiable by about embryonic day 12 (E12), and robust neurogenesis continues postnatally (Farbman, 1994). We therefore examined olfactory tissue in mice treated with tamoxifen from E9.5–10.5, to label progeny arising from ckPCs active during early olfactory neurogenesis (n=8 embryos). Sections through the nasal mucosa of E12.5 mice reveal sporadic labeling of cells in olfactory regions, with neuronal morphology (Fig 1C,D). Utilizing a LacZ reporter mouse (tamoxifen E10.5–12.5, sacrificed at E18.5, n=6 mice) permitted staining of tissue as a whole mount; at E18.5, whole mount specimens reveal a widespread contribution of ckPCs to olfactory regions (Fig 1F). On whole mount examination, reporter-labeled cells localize densely to the turbinates along the lateral nasal wall, which is lined by olfactory neuroepithelium. In representative cryosections, we identify 207 ± 63 (s.d.) reporter-labeled cells per 100 μm olfactory epithelium (n=5 sections). Control Cre (–) littermates (n=8 mice) treated with tamoxifen in parallel and processed for x-gal staining reveal no background label (Fig 1G). As an additional control, olfactory tissue sections from adult *c-Kit*^{CreERT2/+}; *R26R*^{LacZ} mice that never received tamoxifen were processed for x-gal reactivity, and no reporter expression was identifiable (Fig 1H).

Tamoxifen treatment of mice at postnatal day 1–3 and analysis at day 7 (n=8 mice) indicates that ckPCs are also active during ongoing postnatal olfactory neurogenesis (Fig 1E). Confocal microscopy of cryosections from *c-Kit*^{CreERT2/+} mice crossed to GFP Cre reporters reveals widespread labeling of olfactory neurons, with clearly visible GFP (+) dendrites extending apically and ending in dendritic knobs. Also, GFP-labeled cells are present in the basal germinal zone of the neuroepithelium, with the position and morphology of globose basal cells, consistent with their known role as neuronal precursors (Fig 1E). Taken together, these data demonstrate that ckPCs give rise to olfactory neurons during embryonic and postnatal development.

Postnatal ckPC activity was further investigated by treating *c-Kit*^{CreERT2/+}; *R26R*^{LacZ} mice (n=3) on postnatal day 2 with a single dose of tamoxifen and fixing them 1 month later (Fig 2). Interestingly, x-gal staining reveals not only widespread reporter labeling of OE cells across the turbinates and septum (Fig 2A), mainly in the more apical mature neuronal layers, but also scattered cell clusters in the lamina propria with the morphology of Bowman's glands (Fig 2B). Cell phenotypes were confirmed with dual immunohistochemistry and

confocal microscopy. With a 30 day interval between single-dose tamoxifen induction and fixation, nearly all ckPC-derived OE cells are mature neurons, co-labeled by antibody to OMP and situated apical to the immature Gap43 (+) layers (Fig 2C,D). No reporter-labeled cells were found among the CK5 (+) horizontal basal cell layers (Fig 2E). As expected, bgal (+) cells with gland and duct morphology are co-labeled by antibody to CK18, a marker for glands, ducts and sustentacular cells (Fig 2F). These experiments demonstrate that, in the olfactory mucosa, neurons and Bowman's glands normally emerge from the cohort of ckPCs active in the early postnatal period.

Adult c-Kit (+) progenitors are activated by olfactory loss during neuroepithelial reconstitution

We next examined the role of ckPCs in adult olfactory neuroepithelium. Two daily doses of tamoxifen (2 mg/dose) in 6–10 week old *c-Kit^{CreERT2/+};R26R^{LacZ}* double-heterozygous animals (n=3 mice, 5 sections per animal quantified) followed by a 1 week chase resulted in 41 ± 12 (s.d.) labeled cells/0.5 mm epithelium. LacZ (+) cells were found in patchy clusters in the basal regions of the epithelium (Fig 3A). Based on their position and morphology, the labeled cells are immature neurons and globose basal cells. Immunostaining of normal, un-lesioned adult mouse tissue for c-Kit protein expression confirms sparse patchy olfactory basal cell expression (Fig 3B). Adult mice allowed to survive 1 month following the tamoxifen administration (n=2) had generally <10 labeled cells identifiable per entire section. This contrasts sharply with the findings from neonatal mice given tamoxifen postnatal day 2 and examined 1 month later, discussed above (Fig 2). The presence of only rare, scattered ckPC-derived cells in un-lesioned adults at 1 week or 1 month survivals suggests that ckPCs do not function as label-retaining quiescent progenitors, and is consistent with the relatively low turnover of cells in intact, un-lesion epithelium (Carr and Farbman, 1993).

An experimental olfactory lesion model was used to next study the activity of ckPCs in regenerating olfactory neuroepithelium (Fig 3C–G). Mice were treated with a single dose of methimazole, which causes rapid loss of OE, sparing some basal stem cells and lamina propria (Bergman et al., 2002; Leung et al., 2007). Over the next 10–14 days, the epithelium is reconstituted from stem and progenitor cell activity. Methimazole lesioned *c-Kit^{CreERT2/+};R26R^{LacZ}* mice (n=4) were maintained on tamoxifen to track ckPC activity during methimazole-induced epithelial reconstitution, and we examined tissue at 10 days. With this paradigm, there was extensive distribution of bgal labeled cells found throughout the neuronal layers of the new epithelium (Fig 3C,F,G). Both x-gal reaction (Fig 3C) and antibody to bgal (Fig 3F) result in similar labeling patterns. Rarely, areas were noted to also contain labeled cells in the lamina propria (Fig. 3G), consistent with occasional Bowman's gland cells also emerging from c-Kit (+) progenitors, as seen postnatally (Fig 2). Quantification reflects the extensive ckPC activity during lesion-induced olfactory regeneration (Fig. 3E), with 729 ± 145 labeled cells/0.5 mm epithelium (n=4 mice, 5 sections per mouse). Staining of methimazole-lesioned tissue, following a 10 day recovery, with antibody to c-Kit protein reveals that c-Kit-expressing cells are prominent, deep in the epithelium in the basal cell layers (Fig 3D, 4A–D). Rarely, c-Kit (+) sustentacular or microvillar cells are evident (e.g. Fig 3D, arrow). Visualizing the proliferative marker

PCNA with c-Kit protein demonstrates co-localization in most c-Kit (+) cells (Fig 4B–D). These data are consistent with a model in which a subset of GBCs function as ckPCs, activated by neuroepithelial damage to reconstitute the neuronal population.

For signaling via the c-Kit receptor on olfactory ckPCs, the ligand SCF must be expressed in local tissue. SCF can exist in membrane bound or soluble forms, both capable of being active. Prior reports suggest that *SCF* mRNA is produced by sustentacular cells in embryonic olfactory epithelium (Guillemot et al., 1993; Murray et al., 2003). To confirm expression, mouse nasal tissue sections and Western blots were probed for SCF protein expression. During adult epithelial reconstitution following methimazole lesion, labeling by antibody to SCF appears localized in punctate fashion to cytoplasm of sustentacular or microvillar cells (Fig 3E). Also, as expected based on its role in hematopoiesis, SCF appears strongly expressed by a subset of bone marrow stromal cells (arrow, Fig 3E, arrow). By Western blot, a predominate sharp band of expected size at 43 kD is identifiable from neonatal and adult nasal mucosal tissue (Fig 3F). Our protein expression data are consistent with previous RNA in situ hybridization results, and confirm that local sources of adult SCF production are indeed available from olfactory sustentacular and bone marrow mesenchymal cells.

Reconstituting OE was further characterized immunohistochemically. To confirm unambiguously that cells derived from c-Kit-expressing progenitors are neurons, tissue sections from *Kit^{CreERT2/+};R26^RLacZ* mice treated with the methimazole and tamoxifen paradigm were processed to visualize bgal reporter and neuron-specific β -tubulin (Fig 5A–C). Many of the bgal (+) cells, situated among the neuronal layers of the new epithelium, are co-immunolabeled by the TuJ1 antibody against neurotubulin, as expected for newly differentiating olfactory neurons (Schwob et al., 1995; Goldstein and Schwob, 1996). In addition, mice were treated with a pulse of the thymidine analogue BrdU 2 hours prior to death to permit visualization of cells passing through S-phase. GBCs are the predominant actively cycling cell 10 days post-lesion (Huard and Schwob, 1995; Leung et al., 2007). Co-staining with anti-BrdU and anti-c-Kit (Fig 5D–F), we found that $42 \pm 9.4\%$ of c-Kit (+) cells are BrdU (+). Thus, many c-Kit (+) olfactory basal cells appear to function as proliferative GBCs. Also, co-labeling with antibody to c-Kit and antibody to cytokeratin 5, a cell type-specific marker for HBCs in olfactory tissue, reveals that most ckPCs are keratin 5 (–) GBCs situated above the HBC layer (Fig 5G–I). However, rare c-Kit(+)/keratin 5 (+) cells are identifiable in regenerating adult OE post-methimazole (Fig 5G–I, arrow). Occasional co-expression of c-Kit with HBC markers may be a reflection that the receptor is not strictly confined to “downstream” lineage-committed neuronal progenitors.

Experimental selective depletion of the c-Kit (+) population results in failed olfactory neurogenesis

To determine directly if ckPCs are *required* for adult olfactory neurogenesis, we employed a pharmacogenetic technique to induce the selective depletion of the ckPC population (Fig 6). For this approach, we crossed *c-Kit^{CreERT2/+}* mice with conditional *ROSA26^{eGFP-DTA}* animals, which carry a latent *diphtheria* toxin A (DTA) expression cassette (Ivanova et al., 2005; Klein et al., 2013). Tamoxifen treatment induces Cre-mediated excision of a LoxP-

flanked eGFP and transcriptional stop sequence, leading to specific ablation of ckPCs due to *diphtheria* toxin A expression (Fig 6A). When 4–8 week old methimazole-lesioned *c-Kit^{CreERT2/+};ROSA26^{eGFP-DTA}* mice were treated with daily tamoxifen for 10 days, to induce *diphtheria* toxin A in ckPCs, they failed to reconstitute the OE. Probing olfactory mucosal sections with TuJ-1 to visualize neuronal cell bodies and dendrites within the epithelium indicates that the tamoxifen-treated epithelium is thinner and aneuronal in most areas (Fig 6B). Close inspection reveals rare patches of TuJ-1 (+) neurons in the ckPC-ablated epithelium, which could be explained by incomplete methimazole lesion and/or incomplete Cre-mediated DTA expression (Klein et al., 2013). Quantification of TuJ-1 labeling (Fig 6E) indicates 914 ± 57 (SEM) neurons per 0.5 mm epithelial length in control, methimazole lesioned mice (n=3 mice) versus 113 ± 8.8 neurons per 0.5 mm in ckPC-ablated methimazole lesioned mice (n=3 mice, 10 sections per mouse; $p=0.00005$, t-test). Similarly, probing for the expression of olfactory marker protein (OMP), which is produced only by mature, differentiated olfactory receptor neurons, revealed that generation of differentiated neurons is blocked by deletion of the c-Kit expressing population (20 ± 3.5 (SEM) cells/0.5 mm in lesioned and ckPC-ablated versus 141 ± 10 cells/0.5 mm in methimazole-only tissue at 10 day recovery, $p=0.0009$, t-test; Fig 6C–E). These data demonstrate directly that ckPCs are necessary for lesion-induced neurogenesis in adult olfactory neuroepithelium.

We next sought to determine the identity of the cells that are present in the abnormal epithelium in ckPC-depleted mice that have failed to reconstitute olfactory neurons. Tissue sections were probed for other cell type-specific marker expression. Among the epithelial cells present in the thin, abnormal epithelium resulting from *diphtheria* toxin-induced ckPC depletion and methimazole lesion, expression of the transcription factor Pax6 is widely present (Fig 7A,B). Pax6 is normally present in HBCs, a small subset of globose cells, and in sustentacular cells, as well as some Bowman's gland and duct cells (Fig 7A) (Davis and Reed, 1996; Guo et al., 2010); its preserved expression in *diphtheria* toxin-ablated methimazole lesioned epithelium indicates that ckPCs are not absolutely required for the generation of non-neuronal Pax6 (+) lineages. Indeed, sustentacular and gland cells have been shown to arise from multiple lineages (Huard et al., 1998). In addition, labeling with the HBC marker cytokeratin 5 (CK5) appears unaffected in ckPC-depleted methimazole lesioned mice (Fig 7C). The HBC has been shown to act as a reserve stem cell population activated by epithelial damage (Leung et al., 2007). The presence of Pax6 (+)/CK5 (+) horizontal cells during failed neurogenesis in ckPC-depleted mice suggests that the ckPC population acts downstream of the HBC. Extensive sparing of HBCs is unlikely, as methimazole produces reliable lesion with only patchy, rare areas left undamaged (Bergman et al., 2002).

Finally, epithelial thickness measurements reflect a marked reduction in thickness as a result of failed neurogenesis following methimazole lesion in the ckPC-depleted mice; 13.3 ± 0.7 (SEM) μm in the DTA-treated methimazole lesioned group compared to 37.6 ± 1.5 μm in methimazole-only mice at 10 day recovery (see Fig 7D; $p=0.0005$). Tissue from control *c-Kit^{CreERT2/+};ROSA26^{eGFP-DTA}* mice treated with tamoxifen but without methimazole lesion appeared intact histologically, with only slight reduction in thickness measurement (Fig 7D).

This likely reflects the sparse nature of ckPC activity during normal neuronal turnover. Moreover, these controls demonstrate that activation of DTA in c-Kit (+) cells, without methimazole lesion, does not ablate the neuroepithelium. Thickness measurements were obtained from identical regions of turbinates and anterior-posterior coronal sections, to avoid known variations across the nasal mucosa.

DISCUSSION

The present study sought to provide direct evidence for a function for c-Kit expressing progenitor cells in adult olfactory neurogenesis. Given the importance of c-Kit expression in other stem cell niches, and previous evidence that *c-Kit* and its ligand *Steel* (SCF) appear to be expressed in embryonic olfactory mucosa, we reasoned that ckPCs may support adult OE maintenance. Our results regarding ckPC activity indicate that this cell population is required for the maintenance of adult olfactory sensory neurons. C-Kit-specific fate mapping studies indicate that ckPCs indeed produce olfactory neurons during embryonic and postnatal development. In normal, un-lesioned adult mice, ckPCs support ongoing olfactory neuronal turnover with limited activity. However, OE lesion activates ckPCs to reconstitute the neuronal population. In addition, Bowman's gland and duct cells can also arise from ckPCs. By immunohistochemistry, the c-Kit protein expression pattern localizes to proliferative OE basal cells, which appear to be largely GBCs. Importantly, the specific depletion of the ckPC population from adult mice results in a failure of the normal neuroepithelial reconstitution that follows experimental lesion, despite the presence of horizontal basal stem cells. In this model, although the neuronal compartment is not reconstituted, non-neuronal olfactory cell populations are restored in the absence of ckPCs.

The observed results from the DTA-mediated depletion of the c-Kit (+) population could be explained by the expression of the c-Kit receptor on most (or all) of the cells functioning as immediate neuronal precursors, or by the committed transit amplifying population. Alternatively, c-Kit expression by a more upstream multipotential cell could lead to the same results, if other c-Kit (–) progenitors give rise to a majority of the gland and sustentacular cell populations. A distinct gland and duct-derived sustentacular cell lineage has been described previously (Huard et al., 1998), that could contribute to Pax6 (+) cell reconstitution in the absence of ckPCs. Finally, one must consider the possibility that c-Kit (+) cells could support other progenitor cells via intercellular or paracrine signals, and the ablation of the c-Kit (+) population then has indirect effects on OE cell reconstitution. It is possible that a combination of these scenarios exists, leading to the perturbed OE reconstitution that we observe when the ckPC population is experimentally depleted by DTA targeting. Future experiments to determine the mechanisms mediating c-Kit receptor activation in OE cells will help clarify these questions. However, by fate mapping we observe that neurons are the predominate cell type normally arising from c-Kit (+) progenitors following lesion.

C-Kit signaling in other systems

First identified in 1986, the c-Kit receptor has been a subject of substantial interest because of its important roles in stem cells, development, and in certain cancers. The receptor is

expressed in multiple tissues developmentally, however, mouse knockout models do not show loss of function at all sites of expression (reviewed in (Lennartsson and Ronnstrand, 2012)). In adult tissue, hematopoietic stem cells depend on c-Kit signaling for survival and proliferation. It is of particular interest that in the adult rat brain, c-Kit is expressed in neuroproliferative zones, including the subventricular zone and the subgranular zone of the hippocampus (Jin et al., 2002). In the experiments reported here, the use of temporally controlled cre/LoxP-mediated recombination in ckPCs in adult mice enabled us to define the role of c-Kit receptor-expressing cells in olfactory neuroepithelial reconstitution, an important adult function not previously appreciated. More broadly, c-Kit signaling may be a feature of tissues supporting adult neurogenesis, given its expression in adult neuroproliferative zones, and our findings that ckPCs are essential in the most active of these zones, the neuroepithelium in the nose.

The robust self-renewal of adult olfactory neuroepithelium can be considered, in some respects, to be analogous to the self-renewal of hematopoietic cells in bone marrow, which also require c-Kit signaling. The hematopoietic stem and progenitor cells are the best-studied self-renewing system, largely because of techniques permitting their experimental manipulation (i.e. isolation, in vitro expansion, and transplantation assays). Efforts to define the cellular stages involved in hematopoiesis have resulted in identification of precursor cells based on their surface receptor expression profile (Shizuru et al., 2005). Shared expression of key signaling molecules such as c-Kit, Notch, and the Wnt/ β -catenin pathway (Tietjen et al., 2003; Shizuru et al., 2005; Wang et al., 2011), by both hematopoietic and neuroproliferative niches suggests similar regulatory strategies.

C-Kit expression in olfactory basal cells: possible functions

The activation of the c-Kit signaling pathway in OE basal cells could influence cell proliferation, cell fate decision, differentiation and/or cell survival. In the bone marrow stem cell analogy, downstream signaling events arising from SCF stimulation of the c-Kit receptor can utilize the signal transducers and activators of transcription (STAT) 3 and 5 pathways; the phosphatidylinositol 3-kinase pathway activating AKT; and the mitogen-activated protein kinase RAF-MEK-ERK pathway. The absence of an embryonic olfactory phenotype in spontaneous Kit mutants such as the *W* model (Orr-Urtreger et al., 1990) suggests that c-Kit signaling in the OE likely functions in a complex manner, perhaps modulating or influencing other key signaling pathways. Pleiotropic effects from c-Kit activation on bone marrow progenitors indicate that c-Kit signaling can also directly modulate both Notch and Hes activity (Zeuner et al., 2011). It is tempting to speculate that c-Kit could utilize analogous mechanisms in the OE, given the prominent roles of basic helix-loop-helix genes in olfactory renewal (Cau et al., 1997; Manglapus et al., 2004), known expression of Notch1 and Hes5 during OE development (Schwartz et al., 2007), and OE progenitor cell transcriptome data (Tietjen et al., 2003). Experiments on these problems are beyond the scope of the present report, but merit investigation and will be the focus of future study.

We found that SCF, the known ligand for the c-Kit receptor, appears to be expressed by the sustentacular cell layer. This is in agreement with prior mRNA in situ hybridization data

(Guillemot et al., 1993). Direct damage injuries to the OE, such as methimazole or methyl bromide lesion, destroy the sustentacular layer along with neurons, which might deprive the ckPCs of this growth factor. It is of interest that there is intense labeling of turbinate bone marrow cells with anti-SCF. Since SCF may function in diffusible or membrane bound forms, it is possible that bone marrow could provide an alternative soluble SCF source. Also, the gland and duct-derived sustentacular cell lineage (Huard et al., 1998) may rapidly reconstitute a supply of SCF-producing cells, independent of basal cell activity. During epithelial reconstitution, differentiating sustentacular cells can be localized adjacent to GBCs as the new OE cell layers begin to re-emerge (Goldstein and Schwob, 1996), providing a means for membrane bound or soluble SCF to act upon its receptor.

OE basal cells: a complex stem and progenitor cell niche

Although multipotential stem cells have been described among the globose and horizontal basal cell compartments (Goldstein et al., 1998; Huard et al., 1998; Leung et al., 2007), the progenitor cell populations required for olfactory neuron maintenance, and the regulation of these niches, have remained incompletely defined (Guillemot et al., 1993; Gordon et al., 1995; Cau et al., 1997; Manglapus et al., 2004). Notably, disruption of *Ascl1* leads to a marked reduction in neurons but increase in *SCF*-expressing cells in the mutant embryonic epithelium (Murray et al., 2003), suggesting the importance of c-Kit signaling in the developing OE. Our results demonstrate, for the first time, the importance of the c-Kit (+) population also in the adult OE reconstitution process. It is also of interest that, on more recent detailed study, patches of c-Kit (+) cells are identifiable in the embryonic *Ascl1* mutant olfactory mucosa, and they seem to localize to the areas where rare neurons are still produced (Krolewski et al., 2012). Considering the present findings regarding the role of c-Kit expressing cells in adult OE, we propose a model reflecting the requirement for olfactory ckPCs to maintain the adult neuronal population (Fig 8). In this scheme, we incorporate the existing conclusions that both horizontal and globose basal cells can behave as multipotential cells, and that the GBC population is functionally heterogeneous, comprised of quiescent reserve cells, transit amplifying progenitors, and immediate neuronal precursors (Huard et al., 1998; Leung et al., 2007; Gokoffski et al., 2011; Jang et al., 2014). We also note the ability of sustentacular and gland/duct cells to self-renew. Although most c-Kit derived cells in our fate mapping experiments were neurons, we did find non-neural populations labeled, most notably Bowman's glands and ducts (e.g. Fig 2). Therefore, the possibility that c-Kit may at times be expressed by multipotential basal cells is indicated. Indeed, a complex, reversible dynamic among basal cells functioning as stem or progenitor cells has been suggested, reflected by expression of Sox2 and/or *Ascl1*, and regulated by TGF β -superfamily extracellular cues (Gokoffski et al., 2011). The reversible states are reflected by arrows on our schematic figure. Given the expression reported here, feedback signals via SCF from the sustentacular layer acting via c-Kit could contribute in a similar manner to influence stem and/or progenitor cell behavior. Also, such a model does not exclude the possibility that c-Kit may also function in other distinct lineage-committed progenitors.

Translational considerations

By virtue of their anatomic location in direct contact with the nasal airspace, olfactory neurons are inherently vulnerable (Carr and Farbman, 1993). Mammals appear to have retained the capacity for robust self-renewal of the olfactory neuroepithelium, to maintain this critical sensory modality. However, their anatomic location also provides us with the clinical ability to access the olfactory area of the nose with minimal risk (Ronnett et al., 2003; Holbrook et al., 2005), in contrast with other regions such as the subventricular zone of the brain. This raises the prospects of (a) exploiting the neurogenic properties of olfactory tissue or (b) possibly delivering novel therapies directly to olfactory mucosa. However, despite the relative ease with which nasal lamina propria mesenchymal-like cells can be propagated in vitro (Murrell et al., 2005; Goldstein et al., 2013), techniques to efficiently expand OE basal stem and progenitor cells from adult mammals have remained challenging (Krolewski et al., 2011). Thus, efforts to better define the biology of adult olfactory stem and progenitor cells are significant. The identification of the importance of c-Kit (+) OE basal cells in adult olfactory maintenance contributes to this effort.

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Figure 1. ckPC fate mapping during embryonic and postnatal peripheral olfactory development. (A, B) Genetic strategies for the inducible GFP or β gal labeling of progeny from c-Kit-expressing cells. (C, D) Representative section through olfactory region of *c-Kit^{CreERT2/+};GFP* reporter embryo induced with tamoxifen from E9.5–10.5 and killed at E12.5, revealing scattered GFP labeled cells in olfactory epithelium with bipolar neuronal morphology (arrow). Arrowhead marks dendrite ending in a dendritic knob at the nasal airspace (asterisk). (E) In olfactory epithelium from *c-Kit^{CreERT2/+};GFP* reporter mouse, tamoxifen-induced postnatal day 1–3, killed day 7, c-Kit expressing progenitors produce neurons. Arrow marks layer of GFP (+) olfactory neurons; note the dendrites extending apically (double arrow), ending in dendritic knobs. There are several GFP (+) basal cells and immature neurons deep in the olfactory epithelium. Sustentacular cell layer is not labeled. Asterisk marks top of epithelium at the nasal airspace. (F, G) Embryos tamoxifen-induced from E10.5–12.5 killed at E18.5, examined as whole mount view of the anterior head bisected sagittally; anterior is towards right, dorsal is towards top of field. (F) In *c-Kit^{CreERT2/+};R26R^{LacZ}* tissue, note extensive x-gal labeling in olfactory epithelium (OE) region along the turbinates. (G) Negative control littermate *c-Kit^{CreERT2/-/-};R26R^{LacZ}*; x-gal reaction results in no background staining. (H) Control tissues from adult *c-Kit^{CreERT2/+};R26R^{LacZ}* mouse that never received tamoxifen, processed for x-gal reaction, reveal no labeling in olfactory epithelium, confirming absence of non-specific Cre activity. Bar=50 μ m; dashed line marks basal lamina in all images.

Figure 2.

C-Kit expressing progenitors exhibit prominent activity in olfactory tissue during the postnatal period, producing neurons and glands. *C-Kit^{CreERT2/+};R26^RLacZ* mice treated with tamoxifen on postnatal day (PD) 2 to induce bgal expression and fixed at 1 month have a mean of 96.8 ± 23 (s.d.) x-gal (+) cells per 0.5 mm of OE (n=3 mice). **(A)** On low magnification overview of x-gal stained coronal sections, bgal (blue) expression is evident in the OE across the nasal cavity, with reporter-labeled Bowman's glands (arrows) scattered along the septal and turbinate mucosa. **(B)** Examination of boxed region in **A** at high magnification shows detail of a typical x-gal (+) ckPC-derived Bowman's gland acinar unit and duct (arrows), along with labeled ckPC-derived OE cells among the neuronal layers. **(C–F)** Sections were processed for dual visualization of bgal (magenta) and cell type-specific markers (green) to confirm ckPC-derived cell phenotypes. **(C)** Most of the bgal (+) OE cells reside within the OMP (+) mature olfactory neuron layers (bracket) at 1 month following tamoxifen induction. **(D)** The Gap43 (+) immature neuronal layers are deeper in the OE; only a single Gap43 (+)/bgal (+) cell is evident (arrow) in this field. **(E)** CK5 marks the horizontal basal cell layer, which does not contain bgal-labeled cells; nuclei are labeled with DAPI (blue, right panel). **(F)** A bgal (+) gland and duct unit expresses CK18 (arrows); in olfactory mucosa, CK18 is selectively expressed by sustentacular cell bodies at the apical layer and their cytoplasmic process which extend basally, and by the duct and acinar cells of Bowman's glands. Dashed line indicates basal lamina, bar=25 μ m.

Figure 3.

Adult c-Kit expressing progenitor cells are activated during injury-induced epithelial reconstitution. (A, B) Un-lesioned adult *c-Kit*^{CreERT2/+}; *R26R*^{LacZ} mice were treated with tamoxifen for two days to induce bgl expression and then recovered 1 week; (A) nasal tissue sections were reacted with x-gal to permit reporter enzyme visualization (blue label). Arrow indicates a cluster of c-Kit derived cells in basal region, with the position and morphology of globose basal cells and immature neurons, reflecting the sporadic activity of c-Kit (+) progenitors during normal olfactory neuronal turnover. (B) Probing un-lesioned adult olfactory tissue for c-Kit protein expression also reveals sporadic basal cell labeling (arrow). (C, D) Mice were given a single dose of methimazole to induce olfactory lesion, and maintained on tamoxifen during epithelial reconstitution prior to sacrifice 10 days later. X-gal labeled cells are evident throughout the new neuroepithelium (C, blue), and antibody to c-Kit (D) robustly labels cells in the basal germinal zone of the regenerating epithelium, consistent with expression by progenitor GBCs; also a single microvillar or sustentacular cell in this field (arrow) is c-Kit (+). (E) Quantification of c-Kit-derived OE cells from un-lesioned versus 10 day post-methimazole lesioned tissue, *** indicated $p < 0.001$, t-test, $n = 3$ mice. (F, G) Additional examples of adult methimazole lesioned sections; (F) antibody to bgl confirms the specificity of x-gal reactivity. (G) Occasional fields have bgl (+) cells present in the lamina propria (arrow), with the location and morphology of Bowman's glands. Nuclei are labeled by DAPI (blue). Dashed line indicates basal lamina, bar = 100 μm .

Figure 4.

C-Kit and SCF protein expression in regenerating adult OE, at 10 days following methimazole lesion. (A) Low magnification view indicates the general pattern of c-Kit immunoreactivity, scattered along the basal regions of the OE (*arrow*). (B–D) PCNA and c-Kit protein co-localize, indicating c-Kit expression in proliferative basal cells. (E, F) Kit ligand, SCF, is detectable locally. SCF expression in reconstituting olfactory tissue localizes to the sustentacular/microvillar cell layer, and to bone marrow; *bracket* in (E) marks the layer of sustentacular cell nuclei at the apical position of the epithelium. *Arrow* indicates a bone marrow cell with intense SCF label. Cell layers are marked: *Sus*, sustentacular cells; *Neu*, neurons; *BCs*, basal cells. (F) By Western blot probed for SCF, a predominate band of about 43 kD is detected in neonatal (lane a) and adult (lane b) olfactory extracts. Nuclei are labeled by DAPI (*blue*). *Dashed line* indicates basal lamina; *bar*=50 μ m in A and E, 25 μ m D.

Figure 5.

Neurons arising from proliferative *c-Kit* (+) progenitors co-express the Cre reporter and neurotubulin. The experimental model for *c-Kit* fate mapping following olfactory lesion is outlined, *c-Kit*^{CreERT2/+}; *R26RLacZ* mice. (A–C) Cells in reconstituting OE that derived from *c-Kit* (+) progenitors are labeled with antibody to *βgal* (magenta); many *βgal* (+) cells are situated in the neuronal layers of the OE and are co-labeled by TuJ1 (green, arrows), indicating that they are immature neurons. (D–F) Mice were given a single injection of BrdU 2 hours prior to sacrifice, to visualize actively mitotic cells. Co-immunolabeling with antibodies to BrdU (green) and *c-Kit* protein (magenta) indicates that $42 \pm 9.4\%$ (n=8 sections) of *c-Kit* (+) cells are also BrdU (+) (arrows), consistent with many *c-Kit* (+) cells functioning as proliferative GBCs following lesion. (G–I) Reconstituting olfactory tissue 10 days following methimazole lesion was also probed for *c-Kit* protein (red or magenta) and CK5 (green), a marker for HBCs, indicating that most *c-Kit*-expressing cells in the OE are GBCs. In this confocal z-stack image, the solid arrow marks a basal cell adjacent to the basal lamina that co-expresses *c-Kit* and CK5. Dashed arrows mark examples of more typical *c-Kit* (+) cells, which are CK5(-) and are situated in the GBC zone, superficial to the

CK5-labeled horizontal cell layer. Occasional co-expression of c-Kit with HBC markers may be a reflection that the receptor is not strictly confined to “downstream” lineage-committed neuronal progenitors. HBCs often have a reactive pyramidal shape during OE reconstitution following methimazole, rather than their thin, flat morphology when quiescent. *Dashed line* indicates basal lamina; *bar*=25 μm in A–F, 50 μm in G–I; DAPI nuclear stain is *blue* in H, I.

Figure 6.

Depletion of *c-Kit*-expressing cells in adult olfactory mucosa results in failed olfactory neurogenesis. **(A)** Genetic strategy for the selective ablation of the *c-Kit* (+) cell population under temporal control of tamoxifen administration. In *c-Kit*^{CreERT2/+}; *ROSA26*^{eGFP-DTA} mice following tamoxifen administration (TAM), *c-Kit* (+) cells will express *diphtheria* toxin (DTA), causing selective cell death. The methimazole lesion paradigm was combined with DTA-mediated ckPC-specific ablation to test directly the requirement for ckPCs in olfactory regeneration. **(B)** Histologic analysis reveals failed reconstitution in the absence of ckPCs. Epithelium is thinner and poorly reconstituted in tamoxifen-treated tissue (**B**₁); TuJ-1 staining (red) shows marked reduction in olfactory neurons, versus typical abundant labeling of new neurons during epithelial reconstitution in littermate of identical genotype treated with methimazole-only (**B**). (**B**₂, **B**₃) Closer views of boxed regions show detail of TuJ-1 labeled soma, dendrites and dendritic knobs present in normally-regenerating controls, and absent in ckPC-depleted tissue. Bracket marks epithelial height, which is markedly thinner in ckPC-depleted tissue. **(C, D)** Differentiated olfactory neurons, marked by OMP expression (red), are prominent by 10 days post-lesion in normal epithelial reconstitution (**C**, arrows) after methimazole, but nearly absent in the ckPC-depleted epithelium (**D**); cellular debris (asterisks) is present in nasal airspace due to recent lesion. **(E)** Quantification of TuJ-1 labeled and OMP labeled neurons reflects the significant neurogenic failure after lesion in animals lacking ckPCs; *** indicates *p*<0.001, **** indicates *p*<0.0001, t-test, *n*=3 mice. Error bars indicate SEM. MZ=methimazole. Bar=50 μm.

Figure 7.

Non-neuronal cells types are present in ckPC-depleted regenerating olfactory epithelium. **(A)** Non-neuronal sustentacular cells (*Sus*) and HBCs (*BC*) express Pax6 (*brown*), stratified in apical and basal regions in normally-reconstituted epithelium after lesion **(A)**, in control mice treated with methimazole only. Unlabeled Pax6 (–) neuronal layers (*Neu*) are present in the middle regions of the epithelium. **(B)** During failed neurogenesis following methimazole treatment in ckPC-depleted mice, the abnormal OE is predominantly comprised by Pax6 (+) cells, with the Pax6 (–) neuronal layers absent. **(C)** HBCs, also identified by expression of cytokeratin 5 (CK5, *red*) are identifiable in the typical layer adjacent to the basal lamina (*dashed line*) in *c-Kit^{CreERT2/+}; ROSA26^{eGFP-DTA}* mice treated with the lesion and ckPC depletion paradigm, indicating that ckPCs act downstream of HBCs. Bar= 50 μm. **(D)** OE thickness was measured from tissue sections obtained from *c-Kit^{CreERT2/+}; ROSA26^{eGFP-DTA}* mice in different conditions: (1) no treatment (control), (2) daily tamoxifen for 10 days, (3) 10 days following methimazole lesion, or (4) 10 days following methimazole lesion with daily tamoxifen. Importantly, TAM treatment alone in this model results in only minimal decrease in OE thickness. Quantification indicates significant differences in mean epithelial thickness. Error bars indicate SEM; *** indicates p<0.001, t-test, n=3 mice. Tamoxifen (TAM); Methimazole (MZ); No treatment (No Tx).

Figure 8.

Schematic diagram of adult olfactory mucosa and proposed role of ckPCs in neuroepithelial maintenance. The basal germinal zone of the neuroepithelium includes horizontal basal cells (*HBCs*) and globose basal cells (*GBCs*), a subset of which express the c-Kit receptor and function as ckPCs. Complex lineage relationships among stem and progenitor cells support adult epithelial maintenance, producing immature olfactory sensory neurons (*OSNi*), which differentiate into mature bipolar receptor neurons (*OSNm*). Sustentacular and microvillar cells (*Sus/mv*) are situated apically, and Bowman's Gland acinar and duct cells are indicated. *Sus/mv* cells produce Stem Cell Factor (*SCF*), the ligand for the c-Kit receptor. Curved and straight arrows reflect previously described relationships among OE cell types. *BL*=basal lamina.

Table 1

Primary antibody reagents.

Antibody	Immunogen and preparation	Source, species, clonality and catalogue#, RRID	Concentration
anti- β -galactosidase	β -D-galactosidase from <i>E. coli</i>	MP Biomedical, rabbit polyclonal, cat #55976 RRID: AB_10013481	1:1000
anti- β -galactosidase	β -D-galactosidase from <i>E. coli</i>	Abcam, chicken polyclonal, cat #ab9361 RRID: AB_307210	1:500
anti-BrdU	BrdU	BD Biosciences, mouse monoclonal, cat#555627, clone 3D4 RRID: AB_395993	1:30
anti-c-Kit	Synthetic peptide corresponding to the residues surrounding Tyr703 of human c-Kit	Cell Signaling Technology, rabbit monoclonal, cat#3074 RRID: AB_10829442	1:50
anti-c-Kit	Synthetic peptide corresponding to the C-terminus of mouse c-Kit	Santa Cruz, goat polyclonal (M14), cat#sc-1494 RRID: AB_631032	1:100
anti-CK5	Synthetic peptide corresponding to the C-terminus of mouse keratin 5	Abcam, rabbit polyclonal, cat#ab24647 RRID: AB_448212	1:1000
Anti-CK18	Synthetic peptide corresponding to residues of human cytokeratin 18	Abcam, rabbit monoclonal, cat #ab32118, RRID: AB_736394	1:500
Anti-Gap43	Synthetic peptide corresponding to the C-terminus of human Gap43	Abcam, rabbit monoclonal, cat#ab75810, RRID: AB_1310252	1:250
anti-OMP	Olfactory marker protein purified from rat olfactory bulbs	Wako, goat polyclonal, cat#019-22291 RRID: AB_664696	1:500
Anti-Pax6	Synthetic peptide corresponding to the C-terminus of mouse Pax6	Millipore, rabbit polyclonal, cat#AB2237 RRID: AB_1587367	1:2000
Anti-PCNA	Synthetic peptide corresponding to C terminal amino acids 243-261 of Human PCNA.	Abcam, rabbit polyclonal, cat#ab2426 RRID: AB_303062	1:500
anti-SCF	KLH conjugated synthetic peptide derived from human SCF	Bioss Inc, rabbit polyclonal, cat#bs-0545R RRID: AB_10855650	1:100
anti-SCF	amino acids 26-214 of stem cell factor (SCF) of human origin	Santa Cruz, mouse monoclonal (G-3), cat#sc-13126 RRID: AB_628238	1:200
TuJ-1	Microtubules derived from rat brain	Covance, mouse monoclonal, cat#MMS-435P RRID: AB_10063408	1:500