Ascl3 transcription factor marks a distinct progenitor lineage for non-neuronal support cells in the olfactory epithelium

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The olfactory epithelium (OE) is composed of olfactory sensory neurons (OSNs), sustentacular supporting cells, and several types of non-neuronal cells. Stem and progenitor cells are located basally, and are the source of all cell types needed to maintain OE homeostasis. Here, we report that Ascl3, a basic helix-loop-helix transcription factor, is expressed in the developing OE. Lineage tracing experiments demonstrate that the non-neuronal microvillar cells and Bowman’s glands are exclusively derived from Ascl3+ progenitor cells in the OE during development. Following chemically-induced injury, Ascl3 expression is activated in a subset of horizontal basal cells (HBCs), which repopulate all microvillar cells and Bowman’s glands during OE regeneration. After ablation of Ascl3-expressing cells, the OE can regenerate, but lacks the non-neuronal microvillar and Bowman’s gland support cells. These results demonstrate that Ascl3 marks progenitors that are lineage-committed strictly to microvillar cells and Bowman’s glands, and highlight the requirement for these cell types to support OE homeostasis.

The mammalian olfactory epithelium (OE) is a pseudostratified epithelium composed predominantly of olfactory sensory neurons (OSNs), which are generated in the basal region and extend apically to the nasal cavity. They are supported by an apical layer of glial-like sustentacular cells1,2. Scattered throughout the OE are the non-neuronal microvillar cells and Bowman’s glands. Bowman’s glands consist of clustered acinar cells located under the OE in the lamina propria, linked to ducts that span the epithelium to transport mucus to the apical surface1. At least three types of microvillar cells have been described in the OE4. Two types, distinguished by different morphologies, express the transient receptor potential channel M5 (Trpm5)5. The third type is characterized by expression of phospholipase C β2 (PLCβ2), and type 3 IP3 receptor (IP3R3), both involved in calcium-mediated signal transduction, and of CD736,7. The latter microvillar cell type has been identified as the primary source of neuropeptide Y (NPY) in the OE, which binds specific receptors to stimulate proliferation of basal progenitor cells and neurogenesis8,9. Knockout of NPY, or its receptor, results in reduced stem cell proliferation and decreased production of OSNs8,10. Numerous lines of evidence have indicated that the microvillar cells play an important role in OE homeostasis8,11–13.

The OE undergoes constant turnover, which is fueled by basally located proliferative progenitors, and quiescent stem cells14–16. Under normal conditions, a heterogeneous population of active progenitors, known as globose basal cells (GBCs), expressing markers such as Lgr5, Ascl1, c-Kit or SEC8 generates the cell types to maintain the integrity of the OE17–23. In contrast, the multipotent horizontal basal cells (HBCs) are relatively quiescent, and are activated only after extensive lesioning of the OE, which removes both sustentacular cells and GBCs14. Re-activated HBCs can regenerate all cell types in the OE14,24.

Ascl genes, members of the achaete scute-like complex family, are basic helix-loop-helix transcription factors (bHLH), which are expressed in progenitor cells of various tissues at the time of cell type specification. In the OE, Ascl1 is found in a subset of GBCs, which give rise to OSNs and sustentacular cells22. A second family member, Ascl2, is a critical regulator of intestinal stem cell fate and follicular T-helper cell specification25,26. Ascl3, the least...
characterized member of the family, is a marker of progenitor cells in the salivary glands, and Ascl3-expressing precursor cells generate both duct and acinar cells in vitro27–29.

Here we demonstrate that Ascl3 is also expressed in the OE, and marks precursors of the non-neuronal microvillar cells and Bowman’s glands during development and regeneration. We report that Ascl3 expression is activated in progenitors during development, and in a subset of HBCs immediately after injury, which generate all microvillar cells and the Bowman’s glands. Using cell-specific ablation and injury-induced regeneration, we show that in the absence of these cell types, neurogenesis of OSNs is reduced but not blocked, which may be due to the decreased number of GBCs and increase in apoptotic cells. Our data provide new insight into the lineage of non-neuronal support cells, and their requirement for OE homeostasis.

Results
Precursors of non-neuronal support cells express Ascl3 during OE development. To investigate the expression profile of Ascl3 during embryonic development of the OE, we used a previously generated mouse strain carrying a fusion cassette of EGFP and Cre recombinase knocked into the Ascl3 gene locus (Fig. 1A)29. In this strain, EGFP expression is driven by the endogenous Ascl3 promoter. We observed EGFP as early as embryonic day 12.5 (E12.5) in the developing OE (Fig. 1B). EGFP-positive cells were detectable throughout embryonic development, at E14.5, E16.5 and E18.5, in cells localized at the apical region of the developing OE (Fig. 1B). There was no overlap observed between the EGFP-labeled cells and OSNs labeled with antibody to TuJ1.

To follow the fate of the Ascl3-expressing cells found in the embryonic OE, we traced the lineage of these cells, using the Ascl3EGFP-Cre+ strain crossed with the R26tdTomato reporter. In Ascl3EGFP-Cre+/R26tdTomato+ mice, Ascl3-expressing cells, as well as their descendants, will be labeled with the red fluorescent protein reporter (RFP). RFP-positive cells were found in the mature OE, which display the morphology of apical microvillar cells. Their identity was confirmed by co-staining OE sections from adult Ascl3EGFP-Cre+/R26tdTomato+ mice (2 months) with antibodies to RFP, and to cell specific markers. RFP co-localized with the microvillar cell markers PLCβ3 and IP3R3 (Fig. 2A,B). In addition, RFP also co-localized with cells expressing Trpm5, the cation channel specifically associated with a second type of microvillar cells (Fig. 2C). Morphology and cell-specific marker staining rule out that the apically localized RFP-positive cells are sustentacular cells. We conclude that the Ascl3 transcription factor is expressed by the lineage generating all types of microvillar cells.

In addition, RFP labeled structures co-localized with aquaporin 5 (AQP5), a marker for duct cells of Bowman’s glands that span the OE, as well as of basally located acinar cells in the lamina propria (Fig. 2D, arrowheads, Fig. S1A, arrows and arrowheads). In contrast, there was no co-localization between RFP-labeled cells and mature OSNs labeled with OMP (Fig. 2E). Moreover, we found no evidence of RFP-labeling of sustentacular cells, GBCs or HBCs under non-injury conditions. Lineage tracing experiment using the Ascl3EGFP-Cre+ strain crossed with the R26tdTomato reporter gave results consistent with those described above. All labeled cells exhibited the morphology of microvillar cells or Bowman’s glands (Fig. S1B; YFP and RFP channels shown), but other cell types were not labeled. Taken together, we conclude that Ascl3 is activated in progenitors, which exclusively generate the-secretory microvillar cells and Bowman’s glands.

Ascl3 expression is maintained in the NPY+ microvillar cells in the adult olfactory epithelium. Further examination showed that a subset of RFP-positive cells in the adult OE co-localizes with antibody to EGFP (Fig. 3A). The identity of these cells was determined using antibodies to cell type specific markers. Co-localization of antibodies to EGFP and NPY showed that mature NPY+ microvillar cells express Ascl3 (Fig. 3B). However, there was no EGFP expression detected in the Bowman’s glands or Trpm5+ microvillar cells (Fig. 3A). No overlap of EGFP with either OSNs or with cytokeratin 5 (CK5), a marker of the basally located HBCs was found (Fig. 3C,D). In situ hybridization on adult OE confirmed that Ascl3-driven EGFP expression recapitulates the endogenous distribution of Ascl3 mRNA (Fig. 3E). Thus, the expression of Ascl3 is maintained...
Figure 2. Ascl3-expressing cells are precursors of microvillar cells and Bowman’s glands.
Immunohistochemistry was performed on OE isolated from Ascl3EGFP-Cre+ / R26tdTomato+ mice (2 months), using antibodies to tdTomato (RFP) and (A) PLC β2, which marks the apical microvilli of microvillar cells, (B) IP3R3, (C) Trpm5, (D) AQP5 and (E) OMP. RFP expression colocalized with microvillar cell markers: PLC β2 (arrowheads), IP3R3 and Trpm5 (arrowheads) and Bowman’s glands markers: AQP5 (arrowheads). (E) No colocalization was detected between RFP and the mature OSN marker OMP. White asterisks mark Bowman’s gland duct cells. Dotted line indicates basal lamina. Nuclei are stained by DAPI (blue). Scale bars: 25 μm.
in the apical NPY+ microvillar cells of adult OE, but not in the Bowman’s glands or Trpm5+ microvillar cells.

We conclude that Ascl3 expression in progenitors of the latter cell types must occur only transiently during fetal development. Furthermore, in contrast to Ascl121, Ascl3 expression was not detected in the basal region harboring the stem and progenitor cells of the adult OE (Fig. 3B–E).

Knockout of Ascl3 does not alter microvillar or Bowman’s gland cell differentiation. To investigate a possible role for the Ascl3 transcription factor in the differentiation of microvillar cells or Bowman’s glands, Ascl3 deficient mice were generated by crossing Ascl3EGFP-Cre+/heterozygotes, in which the entire Ascl3 coding sequence is replaced by EGFP-Cre recombinase (Fig. 1A)29. Homozygous Ascl3 knockout mice (Ascl3EGFP-Cre+/−; referred to as Ascl3−/−) were viable and survived to adulthood. There were no obvious morphological changes in the Ascl3−/− OE (Fig. S2A), and labeling with antibodies to PLCβ2, Trpm5 and AQP5 revealed that both types of microvillar cells, and Bowman’s glands were present in OE from Ascl3−/− mice at numbers similar to wild-type controls (Fig. S2B). Thus, while Ascl3 expression is activated in a progenitor of microvillar cells and Bowman’s glands, loss of this transcription factor does not block generation or differentiation of these cell types.

Ascl3 expression is activated in HBCs during OE regeneration. The OE undergoes constant turnover under normal homeostatic conditions, and has a remarkable ability to regenerate after severe injury. Complete regeneration of the OE occurs within 2–4 weeks after chemically-induced loss of the entire epithelium. To investigate the origin of regenerated microvillar and Bowman’s gland cells in the adult OE, we used the anti-thyroid drug methimazole, which causes extensive delamination of the OE layer following intraperitoneal injection14,30. Loss of the sustentacular cells activates the quiescent HBC stem cells to regenerate all cell types in the OE14,24. Ascl3EGFP-Cre+ mice (3- to 4-week-old) were given a single injection of methimazole and the OE was subsequently analyzed at specified days post-injury (dpi) (n ≥ 3 per time point). The HBCs were detected with antibody to CK5.
Notably, at 1 dpi, immediately following delamination, expression of the Ascl3-EGFP was co-localized with CK5+ in some HBCs (Fig. 4A), revealing that Ascl3 expression is activated in a subset of HBCs early in the regeneration process. As OE regeneration proceeded, Ascl3-EGFP+ cells became more apically localized, and were no longer detected in the basal region (3 and 14 dpi) (Fig. 4A). To detect all cells derived from the Ascl3-positive HBCs, we used Ascl3EGFP-Cre+/R26tdTomato+/mice, in order to follow RFP-labeled cells derived from HBCs in which Ascl3 was expressed. Consistent with the activation of Ascl3 in some HBCs, single RFP-labeled cells were co-localized with CK5 at 1 dpi in the basal region (Fig. 4B). The RFP-labeled cells were expanded to multi-cellular clusters by 3 dpi. By 14 dpi, most RFP-labeled cells were apically located and resembled both types of microvillar cells, or exhibited the morphology of Bowman's glands that spanned the OE (Fig. 4B, arrowheads). Double staining with antibodies to cell type-specific markers at 28 dpi confirmed that RFP label was co-localized with both PLCβ2/IP3R3+ and Trpm5+ microvillar cell types, as well as with AQP5+ Bowman's glands (Fig. 4D–G). In contrast, mature OSNs stained with OMP antibody did not co-localize with RFP (Fig. 4H). Thus, during OE regeneration, Ascl3 expression is activated in some HBCs that serve uniquely as precursors of microvillar cells and Bowman's glands.

Ablation of Ascl3+ cells causes reduction of neuronal layer. Both microvillar cells and Bowman's glands are important for the maintenance of OE homeostasis3,9,11,13. Our data suggest that all microvillar cells and Bowman's glands are derived from Ascl3-expressing progenitors. To investigate the role of these cell types in the OE, we used cell specific ablation. Ascl3EGFP-Cre+ mice were crossed with Rosa26DTA, which carries
Figure 5. Ablation of Ascl3-expressing cells results in absence of microvillar cells and Bowman’s glands, and decreases GBCs and mature OSNs. (A) Ascl3EGFP-Cre/+ /R26DTA/+ mice, DTA expression is activated only in the Ascl3-expressing cells. OE was isolated from Ascl3+/+/R26DTA/+ and Ascl3EGFP-Cre+/+ /R26DTA/+ mice at 2 months of age. (B) H&E staining showed a significant decrease in thickness of the OE in the Ascl3EGFP-Cre+/+ /R26DTA/+ mice compared to Ascl3+/+/R26DTA/+ mice. (C) Staining with antibody to PLC β2 (arrowheads) in OE from Ascl3+/+ /R26DTA/+ mice and Ascl3EGFP-Cre+/+ /R26DTA/+ mice. (D) Trpm5-positive microvillar cells
are present at the apical surface of the OE (arrowheads) in Ascl3EGFP-Cre+/R26DTA/− mice, but not detected in OE of Ascl3EGFP-Cre+/R26DTA/+ mice. (E) Antibodies to aquaporin 5 (AQP5) mark the apical surface of the duct cells in the Bowman's glands extending through the OE in Ascl3EGFP-Cre+/R26DTA/− mice (arrowheads). Ducts cells of the Bowman's glands were only rarely observed in OE from Ascl3EGFP-Cre+/R26DTA/− mice (arrowhead). (F) Antibodies to Sox2 revealed no difference in number of sustentacular cells at the apical surface of the OE between mice of the two genotypes. The number of Sox2+ GBCs was decreased (arrowheads). (G) p63+ HBC numbers are not changed in Ascl3EGFP-Cre+/R26DTA/− mice. (H) SEC8 antibody labels GBCs near the basal layer of the OE in Ascl3EGFP-Cre+/R26DTA/− mice (arrowheads). Significantly fewer GBCs were detected in OE of Ascl3EGFP-Cre+/R26DTA/− mice. (I) Labeling with antibody to OMP showed a significant decrease in the number of labeled mature OSNs in Ascl3EGFP-Cre+/R26DTA/− mice compared to controls. (J) Antibodies to active caspase-3 showed an increase in number of apoptotic cells in the OE of Ascl3EGFP-Cre+/R26DTA/− mice. (K) Quantified results show significant decrease in the thickness of OE and the numbers of PLCβ2 and Trpm5+ microvillar cells, AQP5+ duct cells of the Bowman gland, but no difference in number of p63+ HBCs in the Ascl3EGFP-Cre+/R26DTA/− mice. Quantification also showed a significant decrease in SEC+ GBCs and OMP+ mature OSNs in the Ascl3EGFP-Cre+/R26DTA/− mice. In addition, an increase of caspase-3+ cells was observed in Ascl3EGFP-Cre+/R26DTA/− mice (arrowheads). N ≥ 3 for Ascl3EGFP-Cre+/R26DTA/− and Ascl3EGFP-Cre+/R26DTA/+. ***P < 0.001. n.s., No significance. Data are shown with mean ± SEM. Dotted line indicates basal lamina. Nuclei are stained by DAPI (blue). Scale bars: (A), 20 μm. (B–H), 25 μm.

Non-neuronal cells play a role in OE regeneration. To further investigate the non-cell autonomous function of Ascl3 descendant cells, we examined OE regeneration in control and Ascl3-DTA mice using the induced injury model. Mice (3- to 4-week-old) were given a single injection of methimazole and the OE was examined after 28 days of regeneration. By 28 dpi, OE regeneration had occurred in both Ascl3 control and Ascl3-DTA mice (Fig. 5F, H). However, OE thickness had increased in the control but did not change in Ascl3-DTA (Fig. 6D). This suggests...
that neurogenesis is initiated in both, but stalls after 7 dpi in the Ascl3-DTA OE. In support of this, at 7 dpi the number of SEC8́+ GBCs was 25% lower in the OE of Ascl3-DTA compared to that of control (Fig. 6E), and remained decreased in OE of the Ascl3-DTA at all subsequent time points (Fig. 6E). In addition, the Ascl3-DTA OE had a slightly higher number of apoptotic cells at 7 dpi, which was significantly increased over control at 14, 21, and 28 dpi (Fig. 6F). Our results indicate that maintenance of GBCs, and perhaps OSNs, is dependent on the presence of microvillar or Bowman's gland support cells. Further investigations using the Ascl3-DTA cell ablation model could help to elucidate the critical factors or interactions generated by these cells.

Discussion

Although specific subsets of GBCs, such as the Ascl1-positive cells, are known to be direct precursors of neuronal cells, the molecular signatures which separate the other cell types in the OE are not yet clear. In this report, we identify a distinct cell lineage marked by transient expression of Ascl3 that is committed exclusively to microvillar cells and Bowman's glands during OE regeneration. Our data provide clear evidence that these non-neuronal cells are derived from progenitors in which Ascl3 expression was transiently activated. Therefore, we expect that Pou2f3 must act in the lineage marked by Ascl3 expression to introduce heterogeneity of the microvillar cells. However, since the number of Trpm5́+ cells does not decrease in Ascl3́−/− mice, the two factors are unlikely to act within the same pathway.

Several types of microvillar cells have been identified in the OE, and are distinguished by expression of either PLCβ2 and NPY, or of Trpm5. Knockout of the Skn-1a (Pou2f3) transcription factor results in loss of Trpm5́+, but not PLCβ2, microvillar cells. Our lineage-tracing results demonstrate that all microvillar cells are derived from progenitors in which Ascl3 expression was transiently activated. Therefore, we expect that Pou2f3 must act in the lineage marked by Ascl3 expression to introduce heterogeneity of the microvillar cells. However, since the number of Trpm5́+ cells does not decrease in Ascl3́−/− mice, the two factors are unlikely to act within the same pathway.

Lineage tracing studies have shown that HBCs and some GBCs can differentiate into multiple cell types, including OSNs, Bowman's glands and sustentacular cells. During development and regeneration of the OE, individual c-Kit́+ progenitors generate microvillar cells, Bowman's glands and OSNs, indicating that c-Kit́+ cells are a common progenitor of neuronal and non-neuronal lineages. In contrast, our results demonstrate that the Ascl3-expressing progenitors do not give rise to OSNs. These data suggest that Ascl3 is activated in a subpopulation of the c-Kit́+ progenitor cells, and that activation of Ascl3 occurs in those cells committed to exclusively generate microvillar cells and Bowman's glands. A recent study suggesting that Bowman's glands and microvillar cells are separate lineages is not inconsistent with our results. Our data cannot determine whether both cell types arise from a single or separate progenitors that each express Ascl3. Moreover, labeling of progenitors in that study was done relatively late after injury (7 dpi), a point at which further cell-specific commitment may have occurred.

Figure 6. Time course of OE regeneration in the absence of non-neuronal support cells. Quantification of PLCβ2́, Trpm5́ microvillar cells, duct cells of AQP5́ Bowman's glands, OE thickness, SEC8́ GBCs and caspase-3́ apoptotic cells from Ascl3́+/−/R26DTÁ−/+ and Ascl3GFP-Cré+/−/R26DTÁ−/+ mice at days 7, 14, 21, 28 post-injury. (A–C) Quantified results showed significantly reduced numbers of PLCβ2́ and Trpm5́ microvillar cells, AQP5́ Bowman gland ducts in the Ascl3-DTA mice at days 7, 14, 21, 28 post-injury. (D) Decrease in the thickness of OE was detected from day 14 dpi during regeneration. (E) Decrease of SEC8́ GBCs was observed in the Ascl3-DTA mice at days 7, 14, 21, 28 post-injury. (F) More caspase-3́ apoptosis cells were observed in the Ascl3-DTA mice at days 7, 14, 21, 28 post-injury. N ≥ 3 for control and Ascl3-DTA mice. *P < 0.05, **P < 0.01, ***P < 0.001. n.s., No significance. Data are shown with mean ± SEM.
We have previously shown that Ascl3 is expressed in the salivary gland during embryogenesis.\textsuperscript{29} Lineage tracing demonstrated that the Ascl3-expressing cells generate both acinar and duct cells, suggesting that they are multilineage progenitors. In this report, we show that Ascl3 expression is detected in developing OE as early as E12.5, and also marks precursors of several cell types. In both tissues, Ascl3 expression in embryonic precursors appears transient. In the adult OE, Ascl3 expression is limited to PLC\(\beta\)/NPY\+ microvillar cells, and is not detected in basal progenitor or stem cells, except after injury. This is similar to our finding in the salivary glands that Ascl3 expression is limited to a subset of duct cells, which may have a specialized function.\textsuperscript{28,29} Knockout of Ascl3 did not alter or block the differentiation of microvillar cells or Bowman’s glands during development or regeneration. As in the salivary gland,\textsuperscript{28} we observed no change in the overall morphology of the OE in Ascl3 knockout mice. Although the progenitor cells in which Ascl3 is transiently activated are clearly required to generate the microvillar and Bowman’s gland cell types, the role of the Ascl3 transcription factor remains unclear.

A role for microvillar cells in regulating or maintaining the OE has been demonstrated through analysis of several gene knockouts, including CFTR, NPY\+ and IP3R\textsuperscript{31}. In all cases, loss of gene function in the microvillar cells affected proliferation and regenerative ability of the basal progenitor cells. It is proposed that microvillar cells coordinate signals from the external environment, and also release signals that regulate proliferation of the progenitor cells.\textsuperscript{27-29} The finding that specific ablation of a cilia gene in the HBCs reduced the number of GBCs and of OSNs after chemically-induced injury supports the idea that the stem and progenitor cells may respond to non-cell autonomous signals derived from cells such as the microvillar cells.\textsuperscript{32} Our data are consistent with this model. We show that complete ablation of both types of microvillar cells, as well as Bowman’s glands, has a striking effect on OE thickness and on the number of GBC progenitors. In contrast to ablation of the c-Kit cell population,\textsuperscript{29} or knockout of Ascl1 in GBCs,\textsuperscript{33} which are required for OSN generation, the ablation of Ascl3\+ cells does not block neurogenesis. In the absence of microvillar cells and Bowman’s glands, both immature and mature neurons are still present in the uninjured and the regenerated OE. Thus, although the microvillar cells provide important signals for neuronal maintenance, their absence does not impair the process of neurogenesis.

While neurogenesis does proceed, absence of the microvillar cells and Bowman’s glands leads to a deficiency in establishing or regenerating a complete OE. Our data suggest that there is no difference in regeneration up to day 7, but that proliferation and differentiation of OSNs lags at later time points. We speculate that the decrease in OE thickness, which reflects a lower number of neurons, is due to loss of signals required for stimulating or maintaining GBC cell proliferation, and preventing OSN death to establish the normal OE thickness. The decrease in GBC numbers and increase in apoptotic cells are consistent with this hypothesis.

In summary, our results demonstrate that transient Ascl3 expression marks progenitors that are strictly lineage-committed to microvillar cells and Bowman’s glands during development and regeneration. Ablation of these non-neuronal supporting cells does not block neurogenesis, but leads to impaired development or regeneration of a complete OE, and supports the idea that microvillar cells and Bowman’s glands participate in non-cell autonomous mechanisms to regulate OE integrity.

Materials and Methods

Animals. All mice were maintained on a C57BL/6\textsuperscript{J} background. Generation of the Ascl3\textsuperscript{EGFP-Cre}\textsuperscript{+} mice was done by replacing exon 2, including the entire coding region, with a fusion cassette encoding nuclear EGFP and Cre recombinase.\textsuperscript{29} The Ascl3\textsuperscript{EGFP-Cre}\textsuperscript{+} mice were crossed with the R26\textsuperscript{GT(Tomato)}\textsuperscript{129S-Gt(Rosa)26Sortm14(CAG-Tomato)Hze/J} reporter strain, R26\textsuperscript{Confetti} \((Gt(Rosa)26Sortm1(CAG-Brainbow2.1)Cle/J)) reporter strain and the inducible aBl2 DTAs \((Gt(Rosa)26Sortm1(DTA)Jpmb/J)\) strain (obtained from Jackson Laboratory). Genotyping was performed with following primers: Ascl3: forward 5′-CCACCCCAGTGCCTCTACACAAAT-3′ and reverse 5′-GTCGCTGGAGAAGGGCAGCAGA-3′.

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Analyze the following sentences for their relevance to the main topic of the text:

- “we observed no change in the overall morphology of the OE in Ascl3 knockout mice.”
- “Although the progenitor cells in which Ascl3 is transiently activated are clearly required to generate the microvillar and Bowman’s gland cell types, the role of the Ascl3 transcription factor remains unclear.”
- “A role for microvillar cells in regulating or maintaining the OE has been demonstrated through analysis of several gene knockouts, including CFTR, NPY+ and IP3R3.”
- “In summary, our results demonstrate that transient Ascl3 expression marks progenitors that are strictly lineage-committed to microvillar cells and Bowman’s glands during development and regeneration.”
- “Ablation of these non-neuronal supporting cells does not block neurogenesis, but leads to impaired development or regeneration of a complete OE, and supports the idea that microvillar cells and Bowman’s glands participate in non-cell autonomous mechanisms to regulate OE integrity.”

These sentences are relevant to the main topic of the text, which is the role of Ascl3 in the development and regeneration of the OE. They discuss the expression and function of Ascl3 in various tissues, the role of microvillar cells and Bowman’s glands, and the impact of Ascl3 knockout on OE development and regeneration. The sentences provide evidence for the hypothesis that Ascl3 is a key regulator of OE development and regeneration.
cooling, sections were blocked with 5% normal donkey serum/1% BSA/0.1% Triton X-100 in PBS for 1 hour, then incubated with antibody in PBS with 1% bovine serum albumin overnight at 4 °C. Cy2- or Cy3-conjugated secondary antibodies (1:500, Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-conjugated anti-chicken antibody (1:100, Invitrogen) were used. Tyramide signal amplification kit (Invitrogen) was used to visualize IP3R3, p63 and Sox2 signal according to manufacturer’s protocol. Nuclei were stained with DAPI (Invitrogen). Sections were mounted in IMMUNE-MOUNT (Thermal Scientific). Fluorescent images were acquired on Leica TCS SP5 system using 40x oil immersion objective with a zoom of 1, 1.5 or 2 and processed using Adobe Photoshop. Figures were assembled using Adobe Photoshop.

**In situ hybridization.** Ascl3 sense and antisense riboprobes were described previously and generated using the digoxigenin-labeling kit (Roche) followed by incubation with Anti-Digoxigenin-AP (Roche). The signal was detected using BM Purple (Roche). All frozen sections of mouse olfactory epithelium used were 10 µm. Images were taken using an Olympus DX41 microscope with a D571 camera, analyzed on DP-BSW-V3.2 software and processed using Adobe Photoshop (Olympus America Inc). Figures were assembled using Adobe Photoshop.

**Olfactory epithelial lesion.** Methimazole (U.S. Pharmacopeial) (50 mg/kg of body weight) in saline was intraperitoneally injected into 3–4 week-old mice (both male and female). Olfactory mucosa was isolated at 1, 3, 7, 14, 21 and 28 days after injury and processed as described. N ≥ 3 mice of each genotype were analyzed for each time point.

**Quantitative analysis.** Thickness of the olfactory epithelium was measured in mice of different genotypes along the septum from 3–6 images at the same anteroposterior level by Image J (NIH) software. To analyze different cell types, 3–6 images were taken from dorsal-mesial, ventral medial, dorsal lateral and ventral lateral regions at the same anteroposterior level. ImageJ software was used to measure the length along the basal surface of the OE from each image (in micrometers). Cell numbers were counted from blindly assigned images of each genotype. Data are shown as mean ± SEM per mm of OE. N ≥ 3 mice of each genotype were analyzed. Results were analyzed using the unpaired student's t-test with GraphPad Prism 5 (GraphPad Software). Statistical significance was set at P < 0.05.

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**Author Contributions**

P.L.W. assisted with study design, performed experimental work and data analysis, prepared the figures and assisted with writing the manuscript. M.V. assisted with study design, performed experimental work, prepared and analyzed data. C.E.O. designed and directed the study, and edited the manuscript.

**Additional Information**

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