Wnt-Responsive Lgr5⁺ Globose Basal Cells Function as Multipotent Olfactory Epithelium Progenitor Cells

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Persistent neurogenesis in the olfactory epithelium provides a unique model to study neural stem cell self-renewal and fate determination. In the olfactory neuroepithelium, globose basal cells (GBCs) are considered to be the direct progenitors of olfactory neurons. However, the study of neurogenesis from GBCs has been impeded by the paucity of GBC-specific markers. Here we report that Lgr5, a recently discovered adult stem cell marker, is exclusively expressed in GBCs in neonatal and adult mice. Lgr5⁺ cells display characteristics of cycling stem cells, including Ki67 expression and EdU incorporation. Lineage tracing analysis demonstrates that Lgr5⁺ GBCs regenerate multiple cell types under normal turnover condition or after olfactory lesion. Furthermore, upregulation or downregulation of Wnt signaling *in vivo* indicates a key role of Wnt signaling not only in maintaining Lgr5⁺ cell proliferation and promoting neuroregeneration, but also in delaying sensory neuron maturation. Together, our observations provided new insights into the dynamics of neurogenesis in the olfactory epithelium.

Key words: globose basal cells; Lgr5; neural stem cells; olfactory epithelium; regeneration; Wnt

Introduction

The olfactory epithelium is organized as a pseudostratified epithelial structure mainly composed of olfactory sensory neurons, apical sustentacular cells, Bowman's gland/ducts, microvillous cells, and neural stem cells (Schwob, 2002). Horizontal basal cells (HBCs) and globose basal cells (GBCs) are considered candidate stem cells in the basal compartment of the olfactory epithelium (Carter et al., 2004; Chen et al., 2004). In defined conditions, HBCs can generate multiple differentiated neuronal and glial progeny *in vitro* (Carter et al., 2004). Fate-mapping studies using an HBC-specific Krt5-Cre strongly support the concept that HBCs are multipotent stem cells that can regenerate GBCs and subsequently give rise to both neuronal and non-neuronal lineages in the olfactory epithelium (Leung et al., 2007). In this scenario, HBCs are relatively quiescent and serve as a reserve stem cell pool, which can be stimulated after severe injury.

GBCs are situated in the basal olfactory epithelium between HBCs and immature olfactory sensory neurons. Among the GBC population, there are committed neuronal precursors, whereas

others are mitotically activated, representing transit-amplifying progenitor cells (Caggiano et al., 1994; Huard et al., 1998). During olfactory epithelial neurogenesis, the Ascl1 (Mash1), Ngn1, and NeuroD1 transcription factors sequentially express in distinct but overlapping stages of GBC differentiation (Cau et al., 2002; Manglapus et al., 2004; Packard et al., 2011a). A subset of GBCs are Ascl1⁺ transit-amplifying cells that primarily give rise to Ngn1⁺ and NeuroD1⁺ immediate neuronal precursors, supporting the notion that the GBCs are a fate-restricted cell population with a neuron-specific differentiation potency (Cau et al., 2002; Guo et al., 2010).

A previous study using retroviral lineage tracing suggests that a multipotent progenitor cell population resides in the basal layer of the olfactory epithelium that contains both HBCs and GBCs (Huard et al., 1998). In addition, sorted GBCs also give rise to the majority of olfactory epithelium cell types after transplantation (Chen et al., 2004). However, definitive evidence that supports the idea that GBCs function as multipotent olfactory epithelium progenitor cells *in vivo* remains missing (Jang et al., 2003; Murdoch and Roskams, 2007).

Here we report that Lgr5, a G-protein-coupled receptor family protein that has been identified as a marker of adult stem cells in multiple tissues and organs (Barker et al., 2007; Jaks et al., 2008; Chai et al., 2012; Shi et al., 2012; Yee et al., 2013), is expressed in the GBCs of the olfactory epithelium. Using a genetic lineage tracing approach, we further demonstrated that Lgr5⁺ GBCs are capable of regenerating multiple olfactory epithelium cell lineages, except HBCs, under normal turnover or after epithelial lesion. In addition, we observed that the generation of new olfactory epithelial cells from Lgr5⁺ GBCs is tightly regulated by the canonical Wnt signaling pathway, which controls both the pro-

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liferation of *Lgr5*⁺ GBCs and the differentiation of their immediate progeny. Together, these findings provide novel insights into the mechanisms underlying olfactory neuroepithelium regeneration.

Materials and Methods

Mice and tamoxifen induction. The *Lgr5*^{EGFP-Ires-CreERT2} knock-in mice (Barker et al., 2007) and *Rosa26-LacZ* reporter mice were obtained from The Jackson Laboratory. To induce Cre recombinase in *Lgr5*^{EGFP-Ires-CreERT2}; *Rosa26-LacZ* mice, tamoxifen (Sigma) was dissolved in corn oil and injected intraperitoneally from postnatal day 3 to 4 (P3–P4; 0.3 mg/g body weight). Animals older than 3 weeks were treated with tamoxifen (0.4 mg/g body weight) for 3–5 consecutive days. Animal experiments were conducted with both male and female mice, and were approved by the Animal Care and Use Committee of the University of Pittsburgh.

RT-PCR. Total RNA was extracted from freshly isolated tissue using NucleoSpin RNA II Kit (Macherey-Nagel). SuperScript III SuperMix (Invitrogen) was used to synthesize first-strand cDNA. cDNA was amplified with Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOne Plus Real-Time-PCR System (Applied Biosystems). *Ascl1* and *Lgr5* mRNA expression was amplified by specific primers (Barker et al., 2010; Fletcher et al., 2011). Two most stable reference genes were selected using geNorm software (Vandesompele et al., 2002). Values of mRNA expression were presented as relative expression normalized to the geometrical mean of selected reference genes.

Olfactory epithelium lesion. Methimazole induces widespread olfactory neuroepithelium damage in a dose-dependent manner (Genter et al., 1995). For injury-induced regeneration experiments, 3- to 4-week-old animals were intraperitoneally injected with methimazole (50 μ g/g body weight) as previously reported (Leung et al., 2007).

5-Ethynyl-2'-deoxyuridine incorporation. Adult mice or pups at P2 were injected with 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) two times (50 μ g/g) at 6 h intervals. EdU incorporation was detected with a Click-iT Alexa Fluor 555 Imaging Kit (Invitrogen).

Immunohistology and immunofluorescence. Anesthetized animals were transcardially perfused with PBS and 4% PFA before the olfactory epithelium tissue was taken. Olfactory epithelium tissues were then post-fixed in 4% PFA in PBS overnight at 4°C. After being washed in PBS, tissues were equilibrated sequentially in 5, 15, and 30% sucrose (older mice were decalcified in 250 mM EDTA) and embedded in OCT with the nose pointing up. Sectioning was initiated from the olfactory bulb side (Leung et al., 2007).

Immunohistological staining of tissue sections or fixed cell samples was performed according to standard protocol. Primary antibodies and their dilutions used in the experiments were rabbit anti-GFP (1:1000; Invitrogen), chicken anti-GFP (1:2000; Abcam), goat anti-ICAM-1 (1:500; R&D), rabbit anti-K14 (1:500; Thermo Fisher), mouse anti-K14 (1:500; Abcam; post fixation 1–2 h on ice for this anti-CK14 staining, overnight fixation will mask the epitope), mouse anti-*Ascl1* (1:100; BD), goat anti-NeuroD1 (1:500; Santa Cruz Biotechnology), mouse anti-Ki67 (1:100; BD), rabbit anti- β -galactosidase (1:4000; Cappel MP Biomedical), mouse anti-GAP43 (1:200; Millipore), goat anti-OMP (1:1000; Wako), mouse anti-CK18 (1:200; Abcam; post fixation 1–2 h on ice for this anti-CK18 staining), rabbit anti-CK18 (1:200; Abcam, ab32118), and mouse anti-Tuj1 (1:100; Millipore). For goat anti-OMP, mouse anti-GAP43, and rabbit anti-CK18 staining, antigen retrieval was performed by microwaving (5 min in 10 mM NaCitrate/0.05% Tween 20, pH 6.0) as previously described (Milho et al., 2012).

Imaging and quantitative analysis. Confocal images were collected with a FV1000 Olympus confocal laser scanning microscope (Olympus). For each antibody, cells in olfactory epithelium sections were counted (Fletcher et al., 2011). *LacZ*⁺ clusters were counted according to the criterion as previously described (Huard et al., 1998). Cell counts were corrected using Abercrombie's formula: Corrected number = Count $\times$ [section thickness/(section thickness + mean nuclei size)] (Abercrombie, 1946). Data were presented as mean $\pm$ SD from three independent experiments.

β -gal (LacZ) staining. Olfactory epithelium tissues were processed as described above, but were fixed in 4% PFA for only 1 h for LacZ staining. Cryosections of olfactory epithelium were stained in staining solution containing 1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ $\cdot$ 3H₂O, 0.02% Nonidet P40, and 2 mM MgCl₂ in PBS overnight at 35°C (Barker et al., 2007).

Flow cytometry cell sorting and differentiation. Olfactory neuroepithelium was dissected from *Lgr5*^{EGFP-Ires-CreERT2} pups at P5. Tissues were dissociated into single cell suspension using the Papain dissociation kit (Worthington). After being incubated with an Alexa Fluor 488-conjugated anti-GFP antibody for 1 h (Invitrogen), cells were washed and filtered through a 70 μ m cell strainer (BD Biosciences). *Lgr5*-positive cells were sorted using the BD FACSAria II machine (BD Biosciences) and cultured in NSC medium (Stem Cell Technology) supplemented with 20 ng/ml EGF, 10 ng/ml bFGF, and 2 μ g/ml heparin. To induce differentiation, Neurobasal medium supplemented with N2 and B27 was added from day 3.

Statistical analysis. Quantified results were expressed as the mean $\pm$ SEM. Two-tailed Student's *t* test was used to analyze data. A value of *p* $\leq$ 0.05 was considered statistically significant.

Results

Lgr5 marks GBCs in the olfactory epithelium

We performed quantitative RT-PCR to investigate the spatial and temporal expression patterns of *Lgr5* mRNA in neonatal and adult olfactory epithelium (Fig. 1A). Expression of *Lgr5* was detected in neonatal olfactory epithelium and at a reduced level in adulthood. Interestingly, the pattern of *Lgr5* expression paralleled the expression of *Ascl1* (a neural progenitor cell marker) at each time point. This expression pattern suggests a potential role of *Lgr5* in olfactory epithelium development and proliferation.

To characterize *Lgr5* expression in more detail, we took advantage of the *Lgr5*^{EGFP-Ires-CreERT2} knock-in mouse. In this strain, the endogenous *Lgr5* promoter controls expression of EGFP and the modified Cre recombinase CreERT2 to faithfully represent the endogenous *Lgr5* expression (Barker et al., 2007). Confocal images showed that the *Lgr5*-driven EGFP signal (referred to as *Lgr5*-EGFP hereafter) was detectable at E12 (data not shown). Around P2, the majority of *Lgr5*-EGFP⁺ cells were restricted to the basal compartment (Fig. 1B), and the number of *Lgr5*-EGFP⁺ cells reached a peak. Notably, the expression of *Lgr5*-EGFP was absent in the olfactory sensory neuron layer. Although the number of *Lgr5*-EGFP⁺ cells decreased gradually over time, *Lgr5*-EGFP signal remained in the basal layer in adulthood (Fig. 1C). The abundance of *Lgr5*-EGFP⁺ cells in neonatal olfactory epithelium and their location in the basal layer are consistent with the properties observed for recognized progenitor cells in olfactory epithelium.

Two main cell populations, HBCs and GBCs, reside in the basal layer. HBCs have a flattened morphology and are located directly above the basal lamina. They express markers including Krt5, Krt14, ICAM-1, and P63 (Holbrook et al., 1995; Carter et al., 2004; Fletcher et al., 2011; Packard et al., 2011b). We therefore performed immunostaining to determine which population expresses *Lgr5*-EGFP in the *Lgr5*^{EGFP-Ires-CreERT2} knock-in mice. The results showed that *Lgr5*-EGFP⁺ cells did not express the HBC markers, K14 and ICAM-1 (Fig. 1D,E). Instead, *Lgr5*-EGFP⁺ cells were localized immediately above the ICAM-1 or K14-positive HBCs, and displayed a distinct round morphology that was more characteristic of the GBC population. Consistent with this, at P2, *Lgr5*-EGFP⁺ cells frequently coexpress *Ascl1* or NeuroD1, markers of GBCs and their immediate progeny differentiated toward the neuronal lineage (Fig. 1F,H). Among the *Lgr5*-EGFP⁺ population, 39.8% of the cells are *Ascl1*⁺ and 54.3% of the cells are NeuroD1⁺ (Fig. 1J). In adults, the number of *Ascl1*⁺ cells in the *Lgr5*-EGFP⁺ population was significantly

reduced in the olfactory epithelium of 3-month-old mice (Fig. 1*G,J*). Likewise, NeuroD1 is rarely observed in GBCs of adult mice (Fig. 1*I,J*). These data suggest that Lgr5 is expressed in GBCs in adult mice.

Lgr5⁺ cells are cycling and maintain olfactory epithelium renewal

Based on the established neural progenitor cell characteristics of GBCs, we hypothesized that some Lgr5-EGFP⁺ GBCs would be cycling progenitor cells that maintain olfactory epithelium renewal, similar to its role in the small intestine and skin epithelium (Barker et al., 2007; Jaks et al., 2008). We observed that at P2, nearly all of the Lgr5-EGFP⁺ cells (91.2% of 2520 cells, $n = 3$) expressed Ki67, a proliferation marker (Fig. 2*A,F*). Similarly, Lgr5-EGFP⁺ cells also maintained proliferation (81.9% of 1180 cells, $n = 3$) in adult mice (Fig. 2*B,F*).

We further validated the activity of Lgr5-EGFP⁺ cell proliferation by EdU (a thymidine analog that labels cycling cells during S-phase) incorporation experiment. As expected, injection of EdU labeled 85.6% (2180 cells, $n = 3$) and 72.6% (2320 cells, $n = 3$) of Lgr5-EGFP⁺ cells in P2 (Fig. 2*C,F*) and adult (Fig. 2*D,F*) mice, respectively. We also examined the fate of Lgr5-EGFP⁺ cell progeny in adult mice with an EdU pulse and chase experiment. A substantial fraction of the EdU-labeled cells migrated apically and lost the Lgr5-EGFP signal after a 2 week chase (Fig. 2*E*), suggesting that progeny of Lgr5-EGFP⁺ GBCs have differentiated and migrated into the neuronal zone of the olfactory epithelium. Together, these observations confirmed that Lgr5-EGFP⁺ GBCs represented actively cycling progenitor cells in normal olfactory neuroepithelium.

Previous studies have demonstrated that GBCs as neural progenitor cells directly contribute to olfactory sensory neuron regeneration (Caggiano et al., 1994; Jang et al., 2003). Transplantation of GBC-2-positive FACS-sorted GBCs into a lesioned, recipient olfactory epithelium suggested a multilineage differentiation potential including all epithelial cell types (Chen et al., 2004). However, the regenerative capacity of GBC in unlesioned olfactory epithelium remains to be further investigated. To track the Lgr5-EGFP⁺ cell fate *in vivo*, we crossed the Lgr5^{EGFP-Ires-CreERT2} mouse to the Rosa26-LacZ reporter strain, which allows us to mark permanently Lgr5-EGFP⁺ cells and their progeny with the LacZ reporter transgene by tamoxifen injection.

We injected double-heterozygous mice with tamoxifen at P3–P4 and then analyzed them 3–4 weeks later. X-gal staining

Figure 1. Lgr5 marks GBCs in the olfactory epithelium. **A**, Quantitative analysis of Lgr5 expression in the olfactory epithelium by real-time PCR. **B, C**, Confocal images of Lgr5-EGFP⁺ cells in the olfactory epithelium of mice at P2 (**B**) and 3 months (3M) of age (**C**). Images presented are from olfactory epithelium coronal sections stained with an anti-EGFP antibody to amplify the endogenous EGFP signal. Lgr5-EGFP⁺ cells in the basal layer are marked by arrowheads. **D, E**, Coimmunohistological staining of EGFP with HBC markers, K14 (**D**) and ICAM-1 (**E**). Lgr5-EGFP⁺ cells always rest directly on the ICAM-1⁺ or K14⁺ HBCs. **F–I**, Coexpression of GBC markers Ascl1 or NeuroD1 with Lgr5-EGFP in P2 (**F, H**) and adult olfactory epithelium (**G, I**). Many Lgr5-EGFP⁺ cells lost Ascl1 expression (**G**), and NeuroD1 could hardly be detected in 3-month-old animals (**I**). Boxed areas are highlighted on the right. Lgr5-EGFP⁺Ascl1⁺ cells and Lgr5-EGFP⁺NeuroD1⁺ cells are marked by arrowheads. **J**, The percentage of Ascl1⁺ and NeuroD1⁺ cells in Lgr5-EGFP⁺ population. The mean $\pm$ SD of three independent experiments is presented. Scale bars: **B–I**, 50 μ m.

Figure 2. Lgr5⁺ cells proliferate and maintain olfactory epithelium renewal. **A, B**, Coexpression of Lgr5-EGFP and the proliferation marker Ki67 in P2 (**A**) and adult (**B**) olfactory epithelium. Lgr5-EGFP⁺ Ki67⁺ cells are marked by arrowheads. **C**, EdU labeling demonstrates that the Lgr5⁺ GBCs are proliferating cells in neonatal pups. **D**, In adult olfactory epithelium, EdU⁺ cells are restricted to the basal layer and mostly are Lgr5-EGFP⁺ GBCs. Pups (P2) or adults (2 months old) were injected with EdU two times and analyzed directly 6 h after the last injection. Arrowheads mark representative Lgr5-EGFP⁺EdU⁺ cells. **E**, In normal olfactory epithelium, EdU⁺ cells migrated apically after a 2 week chase. **F**, The percentage of Ki67⁺ and EdU⁺ cells in Lgr5-EGFP⁺ population. Data are given as mean $\pm$ SD. **G–I**, LacZ⁺ cells exhibit morphology of olfactory sensory neurons (**G, H**), supportive sustentacular cells (**H**), and Bowman's gland and duct cells (**I**). Olfactory epithelium cryosections from Lgr5^{EGFP-Ires-CreERT2}; Rosa26-LacZ mice were stained with X-gal 1 month after tamoxifen induction. The arrowhead in **H** marks a sustentacular cell. Typical morphology of Bowman's gland and duct cells was indicated by arrows and arrowheads (**I**). **J, K**, Confocal images show that β -gal-positive cells coexpress OMP and CK18. Scale bars: **A–E, G–K**, 50 μ m.

showed that LacZ⁺ cells formed clusters and contained the major cell types of the olfactory neuroepithelium (Fig. 2*G–I*). In most cases, LacZ⁺ clusters contained two to six cells and individual cells appeared separate and discrete (Fig. 2*G*). However, compact

Figure 3. *Lgr5*⁺ cells regenerate multiple olfactory epithelium lineages after methimazole injury. **A–C**, Robust proliferation of *Lgr5*-EGFP⁺ cells was activated in the early stage of regeneration. Two-month-old *Lgr5*^{EGFP-Ires-CreERT2} mice were injected with methimazole to induce olfactory epithelium damage. At day 2, *Lgr5*-EGFP⁺ cells were activated to proliferate and usually coexpressed Ki67. At postlesion days 5–7, the number of *Lgr5*-EGFP⁺ cells reached a peak, and abundant proliferation continued to day 14 (**A**). **B**, *Lgr5*-EGFP⁺ cells colabeled with GBC marker *Ascl1* in the very early stage of regeneration, but a significant reduction of *Ascl1*⁺ cells was observed at postlesion day 14 (**B**). **C**, *Lgr5*-EGFP is expressed exclusively by the GBCs that are located above the K14⁺ HBCs. Arrowheads mark *Lgr5*-EGFP⁺Ki67⁺ cells in **A** and *Lgr5*-EGFP⁺*Ascl1*⁺ cells in **B**. **D**, Quantitative analysis of *Lgr5*-EGFP⁺ cells during regeneration. **E**, The percentage of Ki67⁺ and *Ascl1*⁺ cells in the *Lgr5*-EGFP⁺ population. The data are shown as mean $\pm$ SD from three experiments. **F**, Genetic lineage tracing using 1-month-old *Lgr5*^{EGFP-Ires-CreERT2}; *Rosa26-LacZ* mice. **G–I**, *Lgr5*⁺ cells regenerate multiple cell types in the olfactory epithelium. *LacZ*⁺ cells are present in basal layer, olfactory sensory neurons, sustentacular cells (**G**), Bowman's gland and ducts (**H**), and microvillous cells (**I**). **J–L**, Confocal images of olfactory epithelium sections double stained with β -gal and OMP (**J**) or CK18 (**K**, **L**). β -gal signal colocalizes with both OMP and CK18. **M**, No colocalization was observed between β -gal and CK14 in HBCs. **N**, EdU labeling (arrow) remained within *Lgr5*-EGFP⁺ GBCs after methimazole-induced lesion followed by a 2 week EdU chase. The arrowhead marks an *Lgr5*-EGFP⁺EdU⁺ cell. **O**, *LacZ*⁺ cells are retained in basal layer 6 months after tamoxifen induction. Scale bars: **A–C**, **G**, **H**, **J–O**, 50 μ m; **I**, 20 μ m.

clusters containing >6 cells could be detected occasionally. From the apical to the basal layers, *LacZ*⁺ descendants of *Lgr5*⁺ cells presented morphological characteristics of sustentacular cells, olfactory sensory neurons, and GBCs (Fig. 2*H*). In addition, *LacZ*-labeled cells with unique morphology of Bowman's glands and ducts were also detected (Fig. 2*I*). However, no *LacZ*⁺ cells were observed in the most basal layer of the olfactory epithelium. Only a small fraction of *Lgr5*⁺ cells (<1%) was labeled with *LacZ* in our experiments. The frequency of *LacZ*⁺ cells dramatically decreased when tamoxifen was injected at adulthood, and we did not detect the formation of *LacZ*⁺ cluster. The limited labeling efficiency can be explained by the weak expression of *Lgr5* in neonatal and the cell proliferation further slowdown in adult. We observed no recombination in control littermates without tamoxifen treatment as previously reported (Barker et al., 2010).

The multipotent regenerative capacity of *Lgr5*⁺ cells was further confirmed by immunocolocalization with olfactory epithelium cell lineage-specific markers. As evidenced by confocal imaging, *LacZ*⁺ cells were frequently colabeled with the olfactory marker protein OMP, suggesting that *Lgr5*⁺ cells gave rise to

mature olfactory sensory neurons (Fig. 2*J*). In addition, *LacZ*⁺ cells also coexpressed cytokeratin 18 (CK18), a marker of sustentacular cells (Fig. 2*K*). The apical location and distinct morphology further supported the sustentacular cell identification. However, we did not detect *LacZ*-positive cells in the HBC layer (Fig. 2*G–K*). Together, these results suggest that a subset of *Lgr5*⁺ GBCs function as multipotent progenitors that can generate the major cell types of the olfactory epithelium under normal physiological conditions.

Lgr5⁺ cells regenerate olfactory neuroepithelial lineages after methimazole injury

The remarkable regenerative capacity of olfactory epithelium has been well documented. Damage of the olfactory epithelium with the antithyroid drug methimazole has been identified as a reliable olfactory lesion model (Genter et al., 1995; Leung et al., 2007). To identify the characteristics of *Lgr5*⁺ cells after severe lesion, we injected 3- to 4-week-old *Lgr5*^{EGFP-Ires-CreERT2} mice with methimazole and analyzed *Lgr5*-EGFP⁺ cells and their progeny at early stages of regeneration. In striking contrast to nonlesioned con-

trols, robust proliferation of Lgr5-EGFP⁺ cells was activated throughout the neuroepithelium at postinjury day 2 (Fig. 3A,D). During the dynamic process of regeneration, almost all Lgr5-EGFP⁺ cells coexpressed the proliferation marker Ki67. The proliferation of Lgr5-EGFP⁺ cells peaked at days 5–7 and extended to day 14 (Fig. 3A,D,E). As evidenced by double staining, most Lgr5-EGFP⁺ cells (63.5% of 820 cells at day 2; 56.5% of 1050 cells at day 5) coexpressed the GBC marker Ascl1 (Fig. 3B,E). At day 14, the frequency of Ascl1-labeled Lgr5-EGFP⁺ cells was clearly diminished. In addition, Lgr5-EGFP⁺ cells were always restricted to the GBC layer immediately above the K14-positive HBCs without overlap (Fig. 3C). These results demonstrate that the Lgr5-EGFP⁺ GBC population is readily activated to proliferate after olfactory epithelium lesion.

To more directly determine the contribution of Lgr5-EGFP⁺ GBCs to olfactory epithelium lineages, we traced the Lgr5-EGFP⁺ cells after methimazole-induced lesion in *Lgr5^{EGFP-Ires-CreERT2}; Rosa26-LacZ* mice (Fig. 3F). At 30 d after tamoxifen injection, LacZ⁺ clusters are distributed widely in the septum and turbinate neuroepithelium (Fig. 3G). Compared with nonlesioned mice, both the number of LacZ⁺ clusters and the number of cells in each cluster were dramatically increased, suggesting the progeny of Lgr5⁺ cells had undergone extensive proliferation and some of them had divided multiple rounds during regeneration.

As judged by their position and morphology, LacZ⁺ clusters frequently contained GBCs, olfactory sensory neurons, sustentacular cells, and cells of the Bowman's glands and ducts (Fig. 3G–I). Double staining demonstrated that the LacZ-labeled cells generated OMP⁺ mature olfactory sensory neurons (Fig. 3J), CK18⁺ Bowman's glands and ducts (Fig. 3K), and sustentacular cells (Fig. 3L). In the regenerated olfactory epithelium, we did not detect LacZ⁺ cells in the basal layer that displayed HBC morphology or colocalized with K14 (Fig. 3M). These observations strongly suggest that Lgr5⁺ cells are multipotent progenitor cells that contribute to most olfactory neuroepithelial lineages (except HBCs) after extensive depletion.

We observed that Lgr5-EGFP⁺ cells retained EdU labeling for at least 2 weeks after injection (Fig. 3N). These results are consistent with a recent finding that GBCs contain BrdU or EdU label-retaining cells in neonatal or lesioned-recovering olfactory epithelium (Jang et al., 2014). Furthermore, despite the decrease in the number of LacZ⁺ clusters as the neuroepithelium turned over, sporadic LacZ⁺ cells in the GBC layer could be found 6 months after tamoxifen exposure (Fig. 3O), suggesting the character of slow cycling progenitor cells. Together, these observations confirmed that Lgr5-marked GBCs contain not only actively cycling but also relatively quiescent progenitor cells, the latter of which can only be activated by lesion.

Differentiation of Lgr5⁺ cells *in vitro*

Based on their easy accessibility, previous studies have explored the possibility of using olfactory epithelium progenitors as a

Figure 4. Proliferation and differentiation of Lgr5⁺ olfactory epithelium cell *in vitro*. **A–C**, In NSC medium, olfactory epithelium cells formed compact adhesive colonies (**A**) or neurosphere (**B**). The majority of cells in these colonies was Lgr5-EGFP⁺ and coexpressed the proliferation marker Ki67 (**C**). Some Lgr5-EGFP⁺ Ki67⁺ cells are highlighted by arrowheads (**C**). **D–G**, Sorted Lgr5-EGFP⁺ cells generated different cell types of the olfactory neuroepithelium. After differentiation in neural medium for 5 d, progeny of sorted Lgr5-EGFP⁺ cells expressed Tuj1 (**E**), DCX (**F**), and CK18 (**G**), markers of differentiated olfactory epithelium cells. Scale bars: **A**, **B**, **F**, **G**, 50 μm; **C**, **E**, 100 μm.

source of neural stem cells for neural repair (Jang et al., 2008; Krolewski et al., 2011). To better characterize these Lgr5⁺ olfactory stem cells *in vitro*, we dissociated the neuroepithelium from *Lgr5^{EGFP-Ires-CreERT2}* mice at P2 and cultured the cells in NSC medium. Interestingly, the Lgr5-EGFP signal was always present in a high fraction of cells in the flattened-compact colony ($43 \pm 3\%$) or floating neurosphere ($39 \pm 2\%$; Fig. 4A,B). Confocal images revealed the Lgr5⁺ cells were also Ki67⁺, suggesting that the colonies were made up of proliferative cells (Fig. 4C). Lgr5⁺ colonies or neurospheres rarely contained cells that express the HBC marker K14 (data not shown). In addition, Lgr5⁺ colonies are Nestin negative (data not shown), an intermediate filament protein, consistent with recent finding that Nestin marked progenitors only in embryonic olfactory epithelium (Murdoch and Roskams, 2008).

We next performed FACS to isolate Lgr5-EGFP⁺ cells and examined their differentiation potential *in vitro* (Fig. 4D). By 5 d differentiation, Lgr5-EGFP⁺ cells give rise to Tuj1⁺ (definitive neuronal-fate marker; Fig. 4E) or DCX⁺ (Fig. 4F) immature olfactory sensory neurons. In addition, CK18⁺ sustentacular cells can also be detected (Fig. 4G). These results further supported the identification of Lgr5⁺ GBCs as multipotent progenitors.

Wnt signaling regulates Lgr5⁺ cell proliferation and neurogenesis

Self-renewal and differentiation of neural stem cells in the olfactory epithelium are controlled by complex mechanisms (Nicolay et al., 2006; Heron et al., 2013). However, the precise cellular and molecular mechanisms governing the stemness of GBCs have not been defined. The β -catenin-mediated canonical Wnt signaling pathway has been widely implicated in maintaining stem cells in a self-renewing state. Binding of Wnt proteins to cell-surface Wnt receptors triggers a cascade of intracellular events, which results

Figure 5. Wnt signaling maintains *Lgr5*⁺ cell proliferation. **A**, Confocal images of β -catenin staining in the olfactory epithelium. Boxed region was magnified at the bottom. Compared with upper adjacent cells (marked by arrows), *Lgr5*-EGFP⁺ GBCs (marked with arrowheads) have increased nuclear accumulation of β -catenin. **B**, Confocal images of *Lgr5*-EGFP⁺ GBC colony stained with Ki67. Arrowheads mark *Lgr5*-EGFP⁺ Ki67⁺ cells. **C**, Quantification of Ki67 and *Lgr5*-EGFP double positive cells in the presence and/or absence of Wnt3a in culture. **D–F**, Confocal images of the olfactory epithelium stained with antibodies for Ascl1 and β -gal in early stage of regeneration. **G**, Quantification of Ascl1 and β -gal double positive cells in the olfactory epithelium of mice as shown in **D–F**. Wnt activation significantly increased the percentage of *Lgr5*⁺ GBC-derived Ascl1⁺ transit-amplifying neural progenitors (*p = 0.000). **H**, Confocal images of olfactory epithelium stained with antibodies for OMP, Ki67, and β -gal. Deletion of APC maintained *Lgr5*⁺ GBC progeny in a proliferative state in the basal layer 1 month after lesion. **I**, Quantification of β -gal⁺ clusters in the olfactory epithelium of the three mice strains 1 month after injury. Data presented are the numbers of clusters that contains 6 or >6 β -gal⁺ cells (*p = 0.003). Scale bars: **A**, 25 μ m; **B, D–F, H**, 50 μ m. WT, wild-type.

in β -catenin accumulation in the cytoplasm and subsequent nuclear translocation (Clevers and Nusse, 2012). β -Catenin interacts with the Tcf/Lef-family transcription factors to activate Wnt target genes.

Since *Lgr5* was identified originally as a Wnt target (Barker et al., 2007), we examined the expression of β -catenin in the olfactory epithelium. In adults, almost the entire olfactory epithelium stained positive for β -catenin, but a slight accumulation of β -catenin in the nucleus was only detected in the basal layer (Fig. 5A). Interestingly, the cells that accumulated nuclear β -catenin colabeled with *Lgr5*-EGFP, suggesting the activation of Wnt signaling in these cells.

To address whether *Lgr5*⁺ GBCs in the olfactory epithelium are regulated by Wnt signaling, we first evaluated the potential effect of Wnt3a (a canonical Wnt isoform) on *Lgr5*⁺ cell proliferation *in vitro*. As expected, Wnt3a treatment substantially increased the percentage of *Lgr5*-EGFP and Ki67 double positive cells (Fig. 5B,C), consistent with previous observation that Wnt3a enhances neuroblast proliferation in hippocampal progenitor cells (Lie et al., 2005). This result suggested that Wnt signaling is involved in regulating *Lgr5*⁺ GBC proliferation.

We next investigated the role of the Wnt/ β -catenin signaling pathway in regulating the proliferation and neurogenesis of *Lgr5*⁺

GBCs during olfactory epithelium regeneration *in vivo*. We manipulated the Wnt signaling pathway by injecting tamoxifen into the *Lgr5*^{EGFP-Ires-CreERT2}; *Rosa26-LacZ*; *APC*^{flox/flox} mice and the *Lgr5*^{EGFP-Ires-CreERT2}; *Rosa26-LacZ*; β -catenin^{flox/flox} mice after methimazole-induced neuroepithelium depletion. This strategy leads to APC or β -catenin deletion in newborn *Lgr5*⁺ cells (or β -gal-labeled cells) and causes Wnt pathway constitutive activation or blocking, respectively (Clevers and Nusse, 2012). We observed that in contrast to wild-type littermates, upregulation of the Wnt/ β -catenin signaling pathway by APC deletion apparently elevated the number of *Lgr5*⁺ GBC-derived Ascl1⁺/ β -gal⁺ transit-amplifying neural progenitors on postinjury day 14 (74.2% vs 21.9%; Fig. 5D,F,G), suggesting an increased neurogenesis in the early stage of regeneration. Interestingly, downregulation of Wnt/ β -catenin signaling activity by β -catenin deletion caused a dramatic reduction in the number of Ascl1⁺/ β -gal⁺ cells (Fig. 5E). Of note, when the tracing period was extended to 1 month, β -gal⁺ clusters with neuroblast morphology could still be detected in the basal layer and many of these cells coexpressed the proliferation marker Ki67 (Fig. 5H). Furthermore, APC deletion clearly increased the number of β -gal⁺ clusters (Fig. 5I) as well as the number of cells in each cluster (data not shown) on postinjury day 30. Together, these data suggest that Wnt activation stimulates *Lgr5*⁺ GBC proliferation and the proliferation of *Lgr5*⁺ GBC-derived transit-amplifying neural progenitor cells, leading to augmented neurogenesis in the early stage of olfactory epithelium regeneration.

Sustained Wnt activation delays olfactory sensory neuron maturation

We next sought to determine whether Wnt activation affects the differentiation of *Lgr5*⁺ GBC progeny in the latter stage of regeneration. Using the same strategy as described in the above experiment, we manipulated Wnt signaling in *Lgr5*⁺ GBCs following methimazole-induced olfactory epithelium injury and examined its effects on the differentiation of *Lgr5*⁺ GBC progeny into olfactory sensory neurons and sustentacular cells at postinjury day 30. We observed that in wild-type control mice, 69.9% of *Lgr5*⁺ GBC-derived β -gal⁺ cells coexpressed the mature olfactory sensory neuron marker OMP, which mainly is expressed in the upper half of the olfactory epithelium (Fig. 6A,D). Upregulation of Wnt signaling caused by APC deletion resulted in a dramatic reduction in the number of β -gal and OMP double positive mature olfactory sensory neurons (20.6% in β -gal⁺ cells; Fig. 6C,D), accompanied by a significant increase of immature olfactory sensory neurons identified by weak or lack of OMP expression (Fig. 6C; 42.4% OMP weak and 35.7% OMP-negative cells). Consistent with this, in APC-deleted olfactory epithelium, the majority of *Lgr5*⁺ GBC-derived β -gal⁺ cells coexpressed the

immature olfactory sensory neuron marker GAP43 (Fig. 6E–H), suggesting that sustained Wnt activation delayed terminal maturation.

However, downregulation of Wnt signaling caused by β -catenin deletion significantly reduced the number of β -gal⁺ clusters and none of the clusters observed contained >6 β -gal⁺ cells (Fig. 6B,F,J), suggesting that neuroregeneration was almost extinguished by downregulation of Wnt activity. These data are consistent with recent observations that downregulation of Wnt signaling inhibits neuronal production in the embryonic cortex (Munji et al., 2011). On the other hand, APC deletion significantly decreased sustentacular differentiation, with only $1.9 \pm 2.3\%$ of β -gal-labeled cells colabeled with CK18 (Fig. 6K,L), compared with $15.9 \pm 6.7\%$ of β -gal⁺ cells differentiated into CK18⁺ sustentacular cells in wide-type mice (Fig. 6I,L). Together, these observations suggest that the Wnt/ β -catenin signaling pathway plays an important role in regulating Lgr5⁺ GBC progeny terminal differentiation.

We also examined the effect of Wnt activation on Lgr5⁺ GBC differentiation *in vitro*. In differentiation media, sorted Lgr5⁺ cells readily differentiated into TuJ1⁺ neurons (Fig. 6M). However, Wnt3a treatment significantly decreased the number of TuJ1⁺ cells while maintaining Lgr5⁺ cells in an undifferentiated state (Fig. 6M,N), further suggesting that Wnt activation prevents Lgr5⁺ cells from differentiating into olfactory sensory neurons. Together, these results support the notion that Wnt signaling regulates olfactory neuroepithelium regeneration not only by controlling the proliferation/self-renewal of Lgr5⁺ GBCs, but also by affecting their subsequent terminal differentiation.

Discussion

In the peripheral nervous system, the olfactory epithelium has the unique property of continuously regenerating new neurons throughout life, replacing cells lost due to environmental insult or disease (Bermingham-McDonogh and Reh, 2011). The robust neurogenesis and relative anatomic simplicity of the olfactory epithelium provides a useful model to understand adult neurogenesis.

GBCs are the major proliferating population of the olfactory epithelium, replenishing olfactory sensory neurons throughout life (Caggiano et al., 1994; Huard and Schwob, 1995; Weiler and Farbman, 1997; Leung et al., 2007). In the current study, we have demonstrated that Lgr5 marks neural progenitor cells in the GBC layer of the olfactory epithelium. Lgr5⁺ cells represent an active cycling population that is retained in adult olfactory epithelium, reminiscent of observations in gastric, intestinal, and skin epithelium (Barker et al., 2007, 2010; Jaks et al., 2008). In these latter systems, Lgr5 marks a long-term, self-renewing population that

Figure 6. Sustained Wnt activation delays olfactory sensory neuron maturation. **A–C**, Confocal images of olfactory epithelium stained with antibodies specific for OMP and β -gal. Compared with wild-type control (**A**), downregulation of Wnt activity by β -catenin deletion obviously reduced the number and size of β -gal⁺ clusters (**B**). In APC-deleted olfactory epithelium, β -gal⁺ clusters were located in the immature or basal layer, and the majority of these cells was OMP negative or had weak OMP expression as highlighted by arrowheads in **C**. **D**, Quantitative analysis of OMP⁺ olfactory sensory neurons in β -gal⁺ clusters (* p = 0.001). Due to the low frequency of labeling, we did not calculate the β -gal⁺ cells after β -catenin deletion. **E–G**, Confocal images of the olfactory epithelium stained with GAP43 and β -gal. β -gal⁺/GAP43⁺ cells were highlighted by arrowheads in **G**. **H**, Quantification of GAP43⁺ immature neuron in β -gal⁺ population (* p = 0.003). **I–K**, Confocal images of the olfactory epithelium stained with antibodies specific for CK18 and β -gal. Compared with wild-type control mice (**I**), Wnt activation significantly reduced the number of β -gal⁺ sustentacular cells located on the surface layer (**K**). **L**, Quantification of CK18⁺ sustentacular cells that express β -gal (* p = 0.008). **M**, Confocal images of sorted Lgr5-EGFP⁺ GBCs in the presence or absence of Wnt3a. **N**, Quantification of the percentage of TuJ1-positive and EGFP-positive cells in culture (* p = 0.000; ** p = 0.002). Scale bars: **A–C**, **E–G**, **I–K**, 25 μ m; **M**, 50 μ m.

maintains epithelial homeostasis. Although most of the Lgr5⁺ GBCs in the olfactory epithelium are transit amplifying under normal physiological conditions, a small population of long-term renewal cells can also be found after lesioning. These unique dynamic processes of Lgr5⁺ cells closely match previous descriptions of the heterogeneous nature of GBCs (Guo et al., 2010).

Many studies implicate GBCs as a multipotent progenitor. In this study, our *in vivo* lineage tracing and *in vitro* culture data strongly suggest that Lgr5⁺ GBCs serve as multipotent progenitor cells and contribute to the regeneration of multiple olfactory epithelial cell lineages. However, Lgr5⁺ GBC-derived β -gal⁺ HBCs were not detected in our lineage-tracing studies under normal physiological conditions or following olfactory epithelium injury. These observations reinforce the concept that GBCs are generated from HBCs after severe epithelial damage (Leung et al., 2007). Beyond revealing an actively proliferating and multipotent GBC population using the Lgr5 marker, the current experi-

ments do not resolve the origin of these cells, which is worthy of future follow-up studies.

Despite the remarkable life-long regenerative capacity of the olfactory epithelium, severe injury often leads to olfactory sensory decline and loss (Schwob et al., 1995). The identification of Lgr5⁺ GBCs as the major regenerative source within the olfactory epithelium provides us novel therapeutic opportunities. Indeed, recent studies have shown that olfactory progenitors isolated from neonatal epithelium can be efficiently expanded *in vitro* and incorporate into the host epithelium after transplantation (Krolewski et al., 2011). It is therefore of particular interest to investigate the *in vivo* behavior of isolated Lgr5⁺ GBCs after being transplanted into lesioned olfactory epithelium in animal models.

Lgr5 was originally identified as a Wnt target, and it also affects Wnt signaling by interacting with Wnt receptor complex (Barker et al., 2007; Carmon et al., 2011; de Lau et al., 2011). Wnt signaling plays an important role in regulating progenitor proliferation and differentiation in many adult tissues and organs, including the central and the peripheral nervous systems (Lie et al., 2005; Kalani et al., 2008; Munji et al., 2011; Chai et al., 2012). In the neuronal tissues and/or organs, Wnt signaling stimulates neural stem/progenitor proliferation and neurogenesis (Wang et al., 2011; Jang et al., 2013; Seib et al., 2013; Shi et al., 2013). Consistent with these previous studies, we observed that Wnt activation augments Lgr5⁺ GBC proliferation and subsequent olfactory epithelium neurogenesis. Interestingly, by taking advantage of the pseudostratified structure of the olfactory epithelium, we also observed that sustained Wnt activity prevents transit-amplifying neuronal progenitor cells from further differentiating into mature olfactory sensory neurons. These results suggest that delicate balance of progenitor cell proliferation and differentiation during olfactory epithelium neurogenesis is temporally and spatially fine-tuned by Wnt signaling activity. A multiple zinc finger transcription factor, Zfp423/OAZ, has been suggested to participate in regulating the transition from differentiation to maturation in olfactory sensory neurons (Cheng and Reed, 2007). It would be interesting to determine the correlation between Wnt and Zfp423/OAZ.

In summary, we demonstrated that the adult stem cell marker Lgr5 marks a population of GBCs. These Lgr5⁺ GBCs possess multipotent regenerative capacity. The generation of other olfactory epithelium lineages from Lgr5⁺ GBCs is delicately regulated by the Wnt signaling pathway, which not only controls the proliferation of Lgr5⁺ GBCs and their immediate progeny, but also affects their subsequent terminal differentiation. Together, our observations provided new insights into the understanding of the mechanisms underlying olfactory epithelium regeneration.

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