Canonical Wnt signaling promotes the proliferation and neurogenesis of peripheral olfactory stem cells during postnatal development and adult regeneration

Ya-Zhou Wang[1,2,*], Takashi Yamagami[1,2,‡], Qini Gan[1,2,‡], Yongping Wang[1,2,‡], Tianyu Zhao[1,2], Salaheddin Hamad[3], Paul Lott[4], Nikolai Schnittke[5], James E. Schwob[5] and Chengji J. Zhou[1,2,3,4,§]

[1]Department of Cell Biology and Human Anatomy, School of Medicine, University of California, Davis, CA 95616, USA
[2]Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children-Northern California, 2425 Stockton Blvd, Room-602B, Sacramento, CA 95817, USA
[3]Graduate Group in Comparative Pathology, University of California, Davis, CA 95616, USA
[4]Genetics Graduate Group, University of California, Davis, CA 95616, USA
[5]Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, MA 02111, USA
*Present address: Institute of Neurosciences, The Fourth Military Medical University, Xi'an 710032, China
‡These authors contributed equally to this work
§Author for correspondence (cjzhou@ucdavis.edu)

Accepted 14 January 2011
Journal of Cell Science 124, 1553-1563

doi:10.1242/jcs.080580

Summary

The mammalian olfactory epithelium (OE) has a unique stem cell or progenitor niche, which is responsible for the constant peripheral neurogenesis throughout the lifespan of the animal. However, neither the signals that regulate the behavior of these cells nor the lineage properties of the OE stem cells are well understood. Multiple Wnt signaling components exhibit dynamic expression patterns in the developing OE. We generated Wnt signaling reporter TOPeGFP transgenic mice and found TOPeGFP activation predominantly in proliferating $Sox2^+$ OE basal cells during early postnatal development. FACS-isolated $TOPeGFP^+$ OE basal cells are required, but are not sufficient, for formation of spheres. Wnt3a significantly promotes the proliferation of the $Sox2^+$ OE sphere cells. Wnt-stimulated OE sphere cells maintain their multipotency and can differentiate into most types of neuronal and non-neuronal epithelial cells. Also, Wnt activators shift the production of differentiated cells toward olfactory sensory neurons. Moreover, $TOPeGFP^+$ cells are robustly increased in the adult OE after injury. In vivo administration of Wnt modulators significantly alters the regeneration potential. This study demonstrates the role of the canonical Wnt signaling pathway in the regulation of OE stem cells or progenitors during development and regeneration.

Key words: Olfactory epithelial stem cells, Wnt, Sox2, Neurogenesis, Regeneration

Introduction

The olfactory epithelium (OE) offers a unique opportunity to study the postnatal and adult stem cell biology and neural regeneration as a consequence of the robustness of stem cell activation and neuronal production throughout the lifespan of the animal (Calof et al., 2002; Loseva et al., 2009; Murdoch and Roskams, 2007; Schwob, 2002). Two groups of cells within the basal layer of the OE function as neuronal precursors, multipotent progenitors and/or stem cells: the globose basal cells (GBCs) and the horizontal basal cells (HBCs) (Iwai et al., 2008; Jang et al., 2003; Leung et al., 2007). Recent studies have shown that HBCs are multipotent and can generate both neural and non-neural (such as Bowman's gland and duct) olfactory cells from early postnatal throughout adulthood under normal conditions (rarely) or in response to injury, particularly when GBCs are destroyed (Carter et al., 2004; Iwai et al., 2008; Leung et al., 2007). However, the niche signals that control the self-renewal and differentiation of these basal cells are not well understood.

The morphogenetic Wnt signaling pathway is well known for its conserved and significant roles in development and disease (Chien et al., 2009; Clevers, 2006; Grigoryan et al., 2008; Logan and Nusse, 2004; Moon et al., 2004). In the CNS, evidence is also accumulating for a role of Wnt signaling in neural development and neural stem cell function (Ciani and Salinas, 2005; Ille and Sommer, 2005; Lyu et al., 2008; Michaelidis and Lie, 2008; Nordstrom et al., 2002; Sommer, 2004). Postnatal and adult neurogenesis is much more prominent in the OE than in the CNS. Therefore, we hypothesize that Wnt signaling acts as a niche signal for the OE stem cells to maintain their self-renewal potential and/or to promote neurogenesis from OE stem cells during development and regeneration.

Using Wnt reporter mice (TOPgal and BATgal), we and other groups have identified a unique subgroup of olfactory ensheathing cells in the inner olfactory nerve layer of the mouse olfactory bulb, where Wnt signaling might have important roles in olfactory axonal connections and regeneration (Booker-Dwyer et al., 2008; Wang et al., 2008; Zaghetto et al., 2007). However, TOPgal or BATgal signaling activity is weak or absent in the OE, probably because of inactivate integration of the reporter gene in the OE of these transgenic mice. To profile Wnt signaling activity in the OE with an appropriate reporter, we have now generated and report here a new transgenic mouse line TOPeGFP (Tcf optimal promoter-driving enhanced GFP reporter gene), which demonstrates high and specific signaling activity, particularly in the OE. We show that $TOPeGFP^+$ OE cells are $Sox2^+$ basal cells and that the dynamic expression of the Wnt signaling components coincides well with the activation

of the TOPeGFP signal. Particularly, we demonstrate that the TOPeGFP$^+$;Sox2$^+$ cells are major OE stem cells or progenitors and that activation of Wnt signaling can promote both self-renewal and neuronal differentiation in the formation of OE spheres and differentiation assays. In addition, we demonstrate that the in vivo administration of modulators of Wnt signaling can significantly alter the OE regeneration potential in adult mice.

Results

Activation of the TOPeGFP transgene and Wnt signaling genes in postnatal OE

To detect the Wnt-responsive cells in the olfactory epithelium, we generated a new transgenic mouse line TOPeGFP, in which eGFP expression was under the control of six copies of Tcf/Lef1 binding sites (the Wnt-responsive elements) and a minimal TK promoter (Fig. 1A). We found that numerous eGFP$^+$ cells were clustered in the basal layer of entire olfactory epithelium at postnatal day (P)5 (Fig. 1B). To establish how Wnt signaling is activated in OE progenitors, we examined the relative expression level of several representative canonical Wnt signaling components by real-time RT-PCR after fluorescence activated cell sorting (FACS). We found that the expression levels of *Axin2* (a putative common target of canonical Wnt signaling pathway), *Tcf7l2* (previous name *Tcf4*, a Wnt signaling transcription factor), *Fzd9* (a Wnt signaling receptor) and *Wnt3a* (a representative canonical Wnt ligand) in the TOPeGFP$^+$ OE cells were relatively higher than in the TOPeGFP$^-$ OE cells at P5 (Fig. 1C). We also examined the expression of these genes by in situ hybridization after taking direct fluorescence images for TOPeGFP activation (Fig. 1D–H). The expression of *Axin2* was higher in the apical cytoplasm of the clustered HBC-like cells in the basal layer, where TOPeGFP was also highly active (arrows in Fig. 1D–F). *Axin2* and TOPeGFP colocalized in GBC-like cells (arrowheads in Fig. 1D–F), but not in olfactory sensory neurons (OSNs) or in sustentacular cells, which had no TOPeGFP expression. *Tcf7l2* and *Fzd9* showed similar expression patterns to *Axin2*, and colocalized with TOPeGFP activation in the clustered HBC-like and GBC-like cells of the basal layer (Fig. 1G,H). *Wnt3a* expression was broadly scattered in the entire OE, including basal cells, OSNs and sustentacular cells, as well as in some non-OE cells beneath the basal lamina (Fig. 1I). In addition, *Wnt2* expression was high in all OE cells except the sustentacular cells (Fig. 1J). By contrast, the *Wnt7b* signal was heaviest at the luminal surface of the sustentacular cells (Fig. 1K). Interestingly, the expression of a Wnt signaling inhibitor *Sfrp1* was extremely low or undetectable in the basal cells or OSN lineage cells, but was highly represented in both sustentacular cells and cells of the lamina propria (Fig. 1L). The expression patterns of positive and negative Wnt signaling components coincide well with the activation of canonical Wnt/β-catenin signaling revealed by TOPeGFP in the OE of neonatal mice.

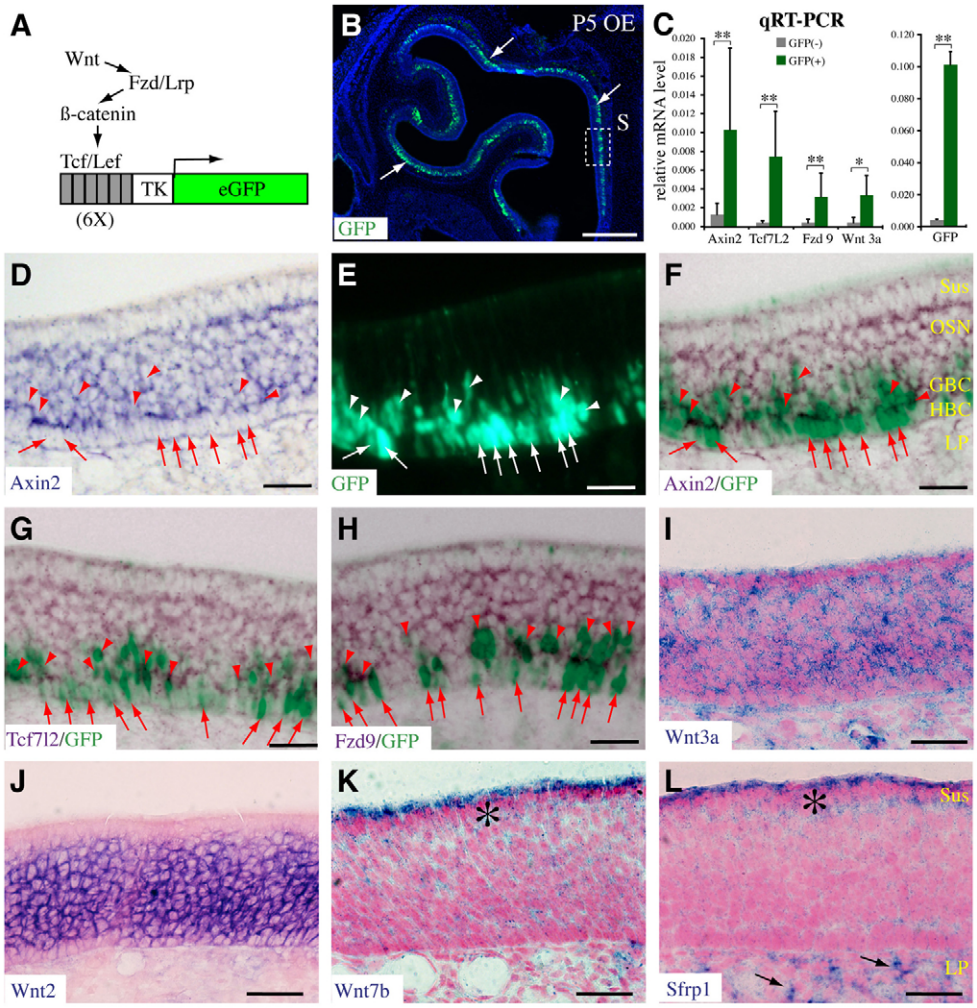

Fig. 1. The expression of TOPeGFP reporter transgene and Wnt signaling components in mouse olfactory epithelium (OE) at P5. (**A**) Schematic illustration of the TOPeGFP transgenic construct. (**B**) eGFP$^+$ cells (arrows) extend throughout the OE in a representative coronal section of the TOPeGFP transgenic mice. The dashed rectangle indicates the septal region of the OE (roughly equivalent to region shown in D–L). (**C**) Real-time RT-PCR for representative Wnt signaling molecules *Axin2*, *Tcf7l2*, *Fzd9* and *Wnt3a* as well as *GFP* in GFP$^+$ and GFP$^-$ cells isolated by FACS from P5 OE tissue of TOPeGFP mice. The relative mRNA level was normalized to *Gapdh*. **P*<0.05; ***P*<0.01. (**D–H**) Colocalization of TOPeGFP (green) with mRNA signals (purple or brown) of *Axin2*, *Tcf7l2* and *Fzd9* in the basal cells (arrows and arrowheads) of P5 OE. The fluorescence microscopy images of eGFP were taken directly (without immunohistochemistry) before the in situ hybridization and inverted to bright fields in F–H. (**I–L**) In situ hybridization demonstrates dynamic expression patterns of representative canonical ligands *Wnt3a*, *Wnt2* and *Wnt7b*, and an inhibitory ligand *Sfrp1* in the P5 OE. Asterisks in K and L indicate the pattern of gene expression in sustentacular cells (Sus). The arrows in L indicate *Sfrp1* expression in the lamina propria (LP). OSN, olfactory sensory neurons. Scale bars: 200 μm (B), 50 μm (D–L).

Lineage and proliferation properties of the Wnt-activated cells in postnatal OE

To determine the cell identity of the TOPeGFP$^+$ cells in the OE, we performed double immunofluorescence with antibodies for eGFP and representative OE lineage markers (Fig. 2). The basal layer of early postnatal OE consisted of a few GBC cell layers and a single layer of HBCs adjacent to the basement membrane. An anti-GBC2 antibody was used to label the GBCs specifically in the basal layer of OE in adult rats (Jang et al., 2003). However, we found that the GBC2 antibody not only moderately labeled TOPeGFP$^+$ basal cells but also intensively labeled the differentiated OSNs in the upper layer of the mouse OE at P5 (data not shown), indicating that the rat GBC2 antibody is not a specific lineage marker for in situ labeling of GBCs in the postnatal mouse OE. Because Sox2 is a crucial embryonic stem cell regulator that is also expressed in the olfactory placode and the related germinal zone during early embryonic development, and has been demonstrated to have important roles in both neocortical neural stem cells and development of the olfactory placode and OE (Cavallaro et al., 2008; Donner et al., 2007), we tested anti-Sox2 antibodies in immunolabeling of OE basal cells (Fig. 2A). We found intense Sox2 immunoreactivity in both OE basal cells (including HBCs and GBCs) and sustentacular cells in the apical layer of the OE, indicating that Sox2 is an optimal marker for both OE basal cells and sustentacular cells. We found that about 60.55±6.55% of TOPeGFP$^+$ basal cells were also positive for Sox2 (Fig. 2A,G). Inversely, about 56.07±2.66% of Sox2$^+$ basal cells were positive for TOPeGFP. Using K5 antibodies to immunolabel HBCs, we found that about 19.06±2.72% of TOPeGFP$^+$ cells were K5$^+$, whereas 50.91±7.65% of K5$^+$ cells were positive for TOPeGFP (Fig. 2B,G). From the acute BrdU immunolabeling (cells incorporated BrdU within 2 hours) for proliferating cells, we found that about 31.8±2.33% of TOPeGFP cells were positive for BrdU (Fig. 2C,G). After 8 hours of BrdU incorporation by four successive administrations of BrdU at 2 hour intervals, the percentage of BrdU-immunolabeled TOPeGFP$^+$ cells was increased to 57.83±6.84% (about 1.8-fold higher than the percentage with acute BrdU incorporation) (Fig. 2D,G). These results indicate that the majority of Wnt-activated OE basal cells are either proliferating or undergoing rapid differentiation by exiting the cell cycle.

By contrast, about 20.88±2.71% of TOPeGFP$^+$ cells were Dcx$^+$ differentiated neuronal progenitors or immature OSNs (Fig. 2E,G). Inversely, only 7.51±1.16% of Dcx$^+$ cells were positive for TOPeGFP. Almost no colocalization was found between TOPeGFP$^+$ cells and Omp$^+$ mature OSNs (Fig. 2F,G). These data further suggest that Wnt signaling is activated dominantly in both proliferating OE basal cells and postmitotic progenitors.

Canonical Wnt signaling enhances primary and secondary OE sphere formation

To address the role of canonical Wnt signaling in the olfactory basal cells, we used an OE sphere assay, which supports the expansion of OE cells in vitro. First, using RT-PCR, we examined whether the Wnt signaling components found in the OE were expressed in the OE spheres. We detected many representative Wnt signaling components in the spheres derived from early postnatal OE; however, *Tcf7* and *Sfrp1* were barely detectable (Fig. 3A). Because sphere formation is an indirect measure of the survival and proliferation of the progenitor cell population(s), we then tested whether manipulation of Wnt signaling can modulate the formation of spheres and TOPeGFP within them. OE cells dissociated from TOPeGFP mice at P3 or P5 were seeded at clonal density and cultured for 7 days under sphere-forming conditions with or without Wnt activators (Wnt3a, BIO, LiCl) or the Wnt inhibitor Dkk1. We observed weak eGFP signals in the primary spheres when no modulators of Wnt signaling were added (Fig. 3B). Stimulation by Wnt3a or BIO or LiCl (with the latter two acting via the inhibition of Gsk3β) dramatically increased

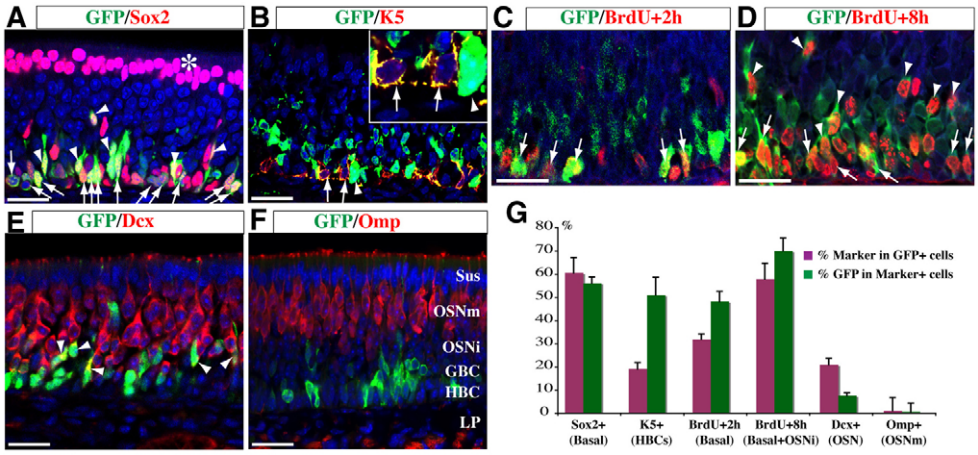

Fig. 2. Double immunolabeling of TOPeGFP with markers for OE basal cells, proliferation and immature or mature OSNs. (**A**) eGFP and Sox2 double immunolabeling. Arrows indicate the colocalization of eGFP and Sox2 (a stem cell marker) immunoreactivity in the HBC layer. Arrowheads indicate the double-labeled cells in the GBC layer. Asterisk indicates the Sox2$^+$/GFP$^-$ sustentacular cell layer. (**B**) eGFP and K5 double immunolabeling. Arrows indicate the colocalization of eGFP and K5 (a HBC marker) immunoreactivity in the HBC layer. Arrowheads indicate a basal cell strongly labeled for eGFP and weak or no K5 immunolabeling. (**C**) Double immunolabeling for eGFP and acute BrdU (2 hour incorporation) in P5 OE. Arrows indicate many double-immunolabeled basal cells. (**D**) Double immunolabeling for eGFP and BrdU (8 hour incorporation from four injections) in P5 OE. Arrows indicate more double-immunolabeled cells in the HBC layer. Arrowheads indicate many double-immunolabeled cells in the GBC and OSN layers. (**E**) Double immunolabeling for eGFP and Dcx (an immature neuronal marker). Arrowheads indicate some double-immunolabeled cells in the GBC and immature OSN layers. (**F**) Most Omp$^+$ mature OSNs are GFP$^-$. (**G**) Percentage of OE basal cells or OSNs (red bars) in TOPeGFP$^+$ cells and percentage of Wnt-responsive cells (green bars) in various OE cell groups immunolabeled with different markers. Cell nuclei were counterstained by DAPI (blue). Data are means ± s.e.m. Scale bars: 50 μm.

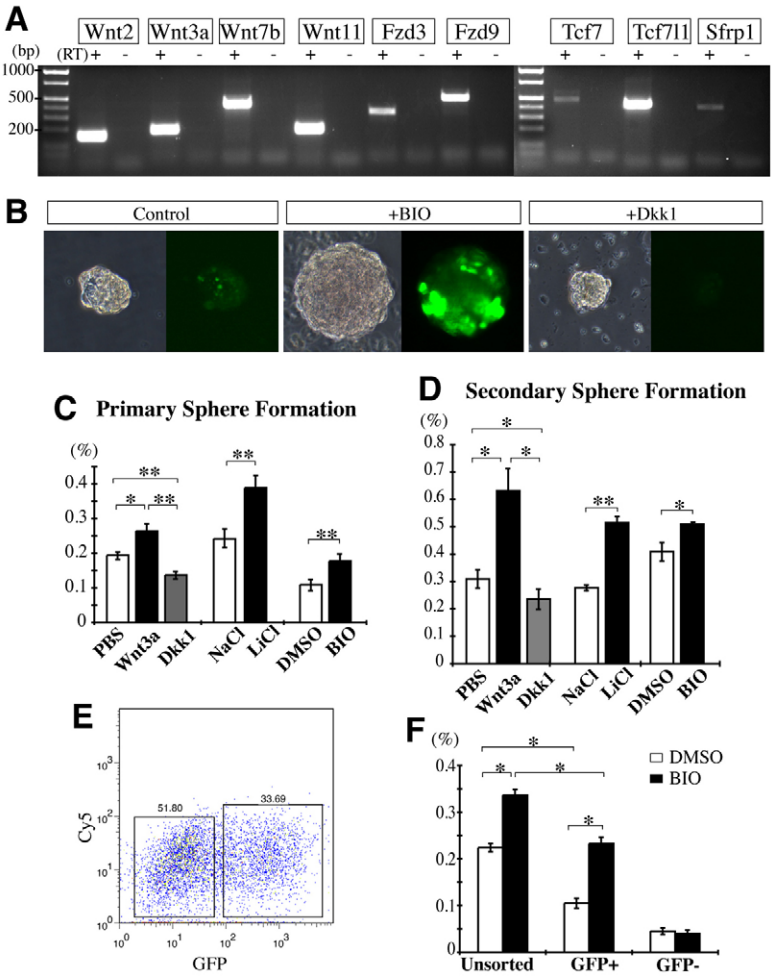

Fig. 3. Wnt signaling promotes primary and secondary OE sphere formation. (**A**) RT-PCR analysis of representative Wnt signaling components in primary OE spheres. The negative controls omitted the reverse transcription step. (**B**) TOPeGFP activation in OE spheres was enhanced by the Wnt activator (Gsk3 inhibitor) BIO and suppressed by the Wnt inhibitory ligand Dkk1 as measured by direct fluorescence microscopy. (**C**) Activators of Wnt signaling (Wnt3a, LiCl, and BIO) increased, whereas Dkk1 inhibited primary OE sphere formation. (**D**) Wnt3a, LiCl and BIO promoted secondary sphere formation, whereas the inhibitor Dkk1 repressed it. (**E**) The GFP gate in FL1 was set with reference to the distribution of unlabeled cells from the wild-type mice for FACS; about 33.69% of the gated cells were GFP^+ in TOPeGFP mice. Cy5 channel (*Y* axis) was used for compensation. (**F**) BIO increased OE sphere formation of both unsorted and GFP^+ sorted cells. The frequency of sphere formation from sorted GFP^+ cells was significantly lower than that obtained from the unsorted OE cells. Very few OE spheres were found in the sorted GFP^- OE cells. $^*P<0.05$; $^{**}P<0.01$.

expression of TOPeGFP in the OE spheres. By contrast, addition of the Wnt antagonist Dkk1 (which blocks Wnt signaling by binding to Lrp5/6) markedly inhibited expression of eGFP (Fig. 3B). With regard to number of spheres, primary sphere formation was increased about 1.4-fold by addition of 5 ng/ml Wnt3a compared with the no Wnt3a control: a statistically significant difference (Fig. 3C). By contrast, primary sphere formation was decreased about 30% upon addition of 400 nM Dkk1, which was also a statistically significant difference compared with the control. In addition, 2 mM LiCl or 500 nM BIO increased primary sphere formation by roughly 1.6-fold compared with their controls (2 mM NaCl or DMSO, respectively). The size of the primary spheres was increased in the presence of LiCl or BIO but not significantly changed by the presence of Wnt3a or Dkk1 compared with their respective controls (data not shown). Interestingly, Wnt3a has been shown to reduce the size of CNS neurospheres (Kalani et al., 2008). These results suggest that Wnt ligands and Gsk3β inhibitors (BIO and LiCl) act through different pathways in the regulation of sphere size.

Because the formation of the secondary spheres might be indicative of the persistence and activation of stem cells, we tested whether Wnt activators could promote secondary sphere formation. We found that secondary spheres formed with similar or slightly higher efficiency when compared with the formation of primary spheres under our culture conditions. Formation of secondary OE spheres was significantly promoted by the Wnt activators Wnt3a, LiCl and BIO, but was repressed by the Wnt antagonist Dkk1 (Fig. 3D).

Canonical Wnt signaling is required, but is not sufficient, to promote OE sphere formation

To further examine the effects of Wnt signaling activation in OE sphere formation, we sorted the $eGFP^+$ and $eGFP^-$ cells from the OE of newborn TOPeGFP mice by FACS (Fig. 3E), and cultured the cells at clonal density with BIO stimulation or DMSO as a control. We found that BIO significantly increased the formation of primary spheres in the sorted $eGFP^+$ group, but the number of spheres formed by the $eGFP^+$ cells (with either BIO or DMSO) was significantly less than those of the unsorted group (Fig. 3F). These results suggest that activation of canonical Wnt signaling is required, but is not fully sufficient, to promote OE sphere formation. In the sorted $eGFP^-$ group, spheres seldom formed, even with BIO stimulation. However, we could not exclude the possibility that a nonepithelial multipotent cell niche (Tome et al., 2009) was contaminated in the $eGFP^-$ and unsorted cells to form some spheres.

Canonical Wnt promotes proliferation of dissociated $Sox2^+$ OE sphere cells

To elucidate the cellular mechanisms of the enhanced neural sphere formation by Wnt signaling activators, we examined the effects of Wnt stimulation on the proliferation and apoptosis of dissociated OE sphere cells. Acute BrdU incorporation showed that most $BrdU^+$

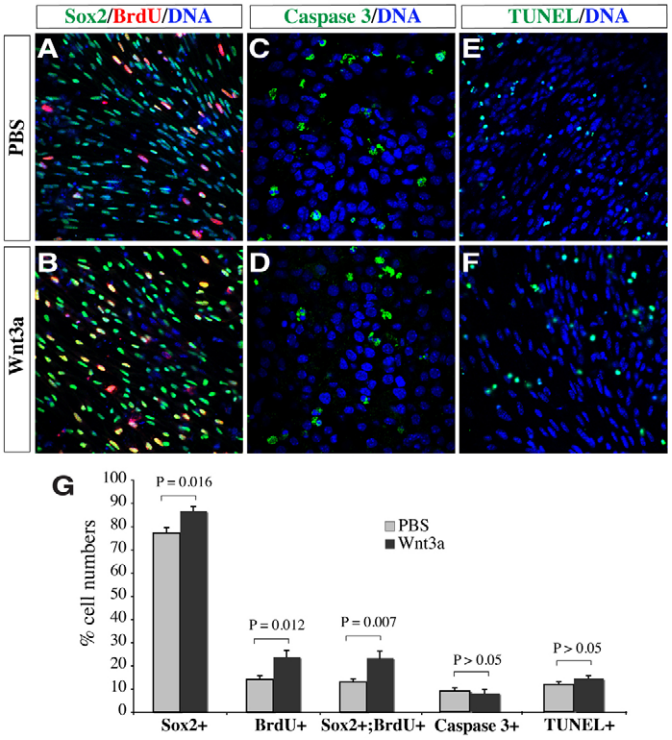

Fig. 4. Effects of Wnt3a stimulation on the proliferation and apoptosis of dissociated OE sphere cells. (**A**,**B**,G) The percentage of $Sox2^+$, $BrdU^+$ or $BrdU^+Sox2^+$ OE sphere cells was significantly increased by Wnt3a stimulation compared with PBS control culture. (**C**–**G**) No significant differences of the percentage of $Caspase3^+$ or $TUNEL^+$ apoptotic cells existed between the Wnt3a treated and control cultures. $P<0.05$ is considered statistically significant.

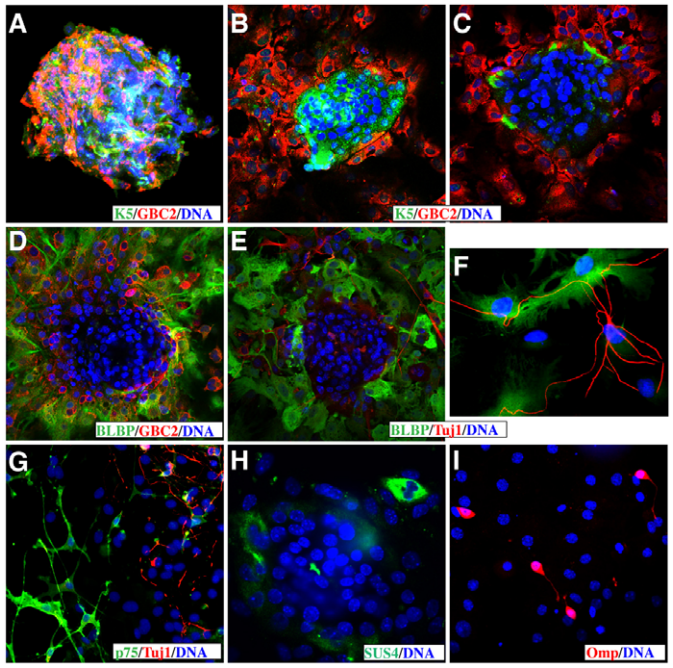

Fig. 5. Multipotency of OE spheres cultured with Wnt activators. (**A**) An undifferentiated adherent OE sphere co-immunolabeled with K5 (for HBCs) and GBC2 (for the GBC lineage). (**B**–**I**) Differentiated adherent sphere cells immunolabeled with lineage-specific marker antibodies including K5 and GBC2 (B,C), BLBP (for olfactory glial lineage) in combination with GBC2 (D), BLBP in combination with Tuj1 (olfactory neuronal lineage) (E,F), p75 (OECs) double immunolabeling with Tuj1 (G), SUS4 (for sustentacular cells) (H) and Omp (for mature OSNs) (I). Cell nuclei were counterstained with DAPI.

cells were $Sox2^+$ cells, and they consisted of about 12% and 77%, respectively, of total dissociated OE sphere cells under the control culture condition (Fig. 4A,G). With Wnt3a treatment, the percentages of the $BrdU^+$ or $BrdU^+Sox2^+$ double-immunolabeled proliferating cells significantly increased about twofold compared with the control (Fig. 4B,G). The percentage of $Sox2^+$ cells was also slightly increased by Wnt3a treatment. Immunolabeling for cleaved Caspase-3 showed that about 8% cells were $Caspase\text{-}3^+$ apoptotic cells in the control culture. No significant change in the number of $Caspase\text{-}3^+$ cells was identified in Wnt3a-treated sphere cell cultures (Fig. 4C,D,G). In addition, the percentage of $TUNEL^+$ apoptotic cells (about 12% in total under the control culture conditions) did not change significantly after stimulation of Wnt signaling (Fig. 4E–G). These data suggest that canonical Wnt signaling promotes OE sphere formation by increasing proliferation or self-renewal, but not by promoting the survival of $Sox2^+$ OE basal cells.

OE sphere cells sustain multipotency after stimulation of Wnt signaling

The multipotent progenitors of the OE differentiate into several olfactory lineages. We tested whether Wnt signaling activation could influence the differentiation of OE stem cells or progenitors in vitro. First, we examined the cell identity of the OE spheres before their terminal differentiation, and found that the OE progenitors in all examined undifferentiated adherent spheres were co-immunolabeled with the OE basal cell markers K5 and GBC2 (Fig. 5A). We then asked whether Wnt activation could sustain the multipotency of OE stem cells. OE spheres were cultured at high density with the presence of Wnt3a, LiCl or BIO for 7 days, and then cultured in differentiation medium with Wnt activators for another 7 days. We found that a variable number of $K5^+$ cells was located in the center of the flattened OE sphere, which were surrounded by numerous $GBC2^+$ cells (Fig. 5B,C). This arrangement resembles the positional relationship between HBCs and GBCs in vivo. BLBP (brain lipid binding protein) is found in OECs and other glial lineage cells. $BLBP^+$ cells with numerous processes were abundant and intermingled with $GBC2^+$ cells; many $BLBP^+$ cells formed a shell around the periphery of $GBC2^+$ cells (Fig. 5D). $Tuj1^+$ (anti-neuron-specific class III β-tubulin) neurons were also located toward the periphery of the flattened sphere (Fig. 5E), and their neurites or axons passed among the $BLBP^+$ cells (Fig. 5F). The neurotrophin receptor p75 is a specific marker for a major subgroup of OECs located in the lamina propria and outer olfactory nerve layer. We found that $p75^+$ OECs were also generated within the OE spheres and migrated to the margins of the differentiated colonies (Fig. 5G). A small number of $SUS4^+$ (a marker for sustentacular cells) or Omp^+ (a marker for mature OSNs) cells were also found in the differentiated spheres (Fig. 5H,I). These results suggest that OE spheres treated with Wnt activator are able to differentiate into most olfactory lineage cells; the migration and distribution of these multiple lineage cells partially mimic their intercellular relationships in vivo.

Canonical Wnt signaling promotes OE neurogenesis

One major feature of OE stem cells is their persistent neurogenesis in vivo. Conditioned medium containing Wnt3a has been shown to promote neuronal differentiation in vitro in embryonic neural stem cells derived from E11.5 mouse telencephalon (Muroyama et al., 2004). We then explored whether activation of Wnt signaling could affect neuronal production from the OE stem cells in the differentiation medium in the presence of Wnt activators. We found that the treatment with Wnt3a, LiCl and BIO during differentiation significantly increased the percentage of Tuj1$^+$ neurons by 3.6-, 3.0- and 5.4-fold, respectively, when compared with the control (Fig. 6A,B). To clarify whether the increased neuronal differentiation was at the expense of non-neuronal differentiation, we also analyzed the percentage of BLBP$^+$ cells. We found that the percentage of BLBP$^+$ cells was reduced significantly in all treatment groups compared with their controls (Fig. 6C,D). Taken together, the results suggest that Wnt signaling promotes OE neurogenesis.

Number of TOPeGFP$^+$ basal cells increases upon methimazole-induced OE regeneration

It is well known that a stem cell or progenitor niche in the adult OE maintains the high regeneration potential under both physiological and pathological conditions (Murdoch and Roskams, 2007; Schwob, 2002). Particularly, the adult OE can be fully regenerated from the stem cells or progenitors after injury. To explore the role of canonical Wnt signaling during induced regeneration, we examined TOPeGFP activities in adult OE with or without drug treatment. An antithyroid pharmacological agent methimazole is highly toxic to the OE and is used widely to induce olfactory regeneration in rodents (Bandyopadhyay et al., 2002; Bergman et al., 2002; Bergstrom et al., 2003; Booker-Dwyer et al., 2008; Brittebo, 1995; Genter et al., 1995; Sakamoto et al., 2007). In the intact or normal control TOPeGFP adult mice administrated with saline intraperitoneally (i.p.), we found a relatively small number of TOPeGFP$^+$ OE basal cells unevenly scattered in all nasal turbinates (Fig. 7A–A″). At 3 days post i.p. injection of methimazole, the TOPeGFP$^+$ OE basal cells were increased significantly in the inferior and superior turbinates and moderately in the middle turbinate and clustered in the septum (Fig. 7B–B″). The thickness of the injured OE was reduced about half (Fig. 7B′,B″), in which the mature OSN and sustentacular cell layers were likely degenerated completely as some residual tissue mass filled in the nasal cavity (asterisks in Fig. 7B). At 7 days post injection, the number of TOPeGFP$^+$ OE basal cells expanded further in all turbinates and in the septum (Fig. 7C). The upper OE layers were partially regenerated with some TOPeGFP$^+$ cells in the sustentacular cell layer (Fig. 7C′,C″). At 14 days post injection, the number of TOPeGFP$^+$ OE basal cells reached a peak in the septum but decreased significantly in the superior and middle turbinates (Fig. 7D). The thickness of the injured OE was still not fully restored, but the sustentacular cells and the surface mucous layer were more clearly recovered at this stage (Fig. 7D′,D″). These results indicate that canonical Wnt signaling actively responds to OE injury and might be required for the rapid expansion of the OE stem cells or progenitors during forced regeneration.

Wnt signaling activation is required for regeneration of adult OE after injury

To further determine the role of Wnt signaling in forced OE regeneration, we applied small molecules of Wnt modulators to the above-described OE regeneration procedure. Quercetin, a plant-derived flavonoid that is used as a nutritional supplement, also a well-known anti-tumor agent (Jagtap et al., 2009; Murakami et al., 2008), is a Wnt inhibitor that acts by disrupting the transcriptional activity of the β-catenin–Tcf complex (Gelebart et al., 2008; Park et al., 2005; Roman-Gomez et al., 2007). Intraperitoneal administration of quercetin significantly prevented the methimazole-induced OE regeneration (Fig. 8A–E). At 2 weeks post methimazole or quercetin treatment, we detected significant decrease of about 71% in Omp$^+$ OSNs, 65% in BrdU$^+$ progenitors and 39% in TOPeGFP$^+$ basal cells in the septal OE

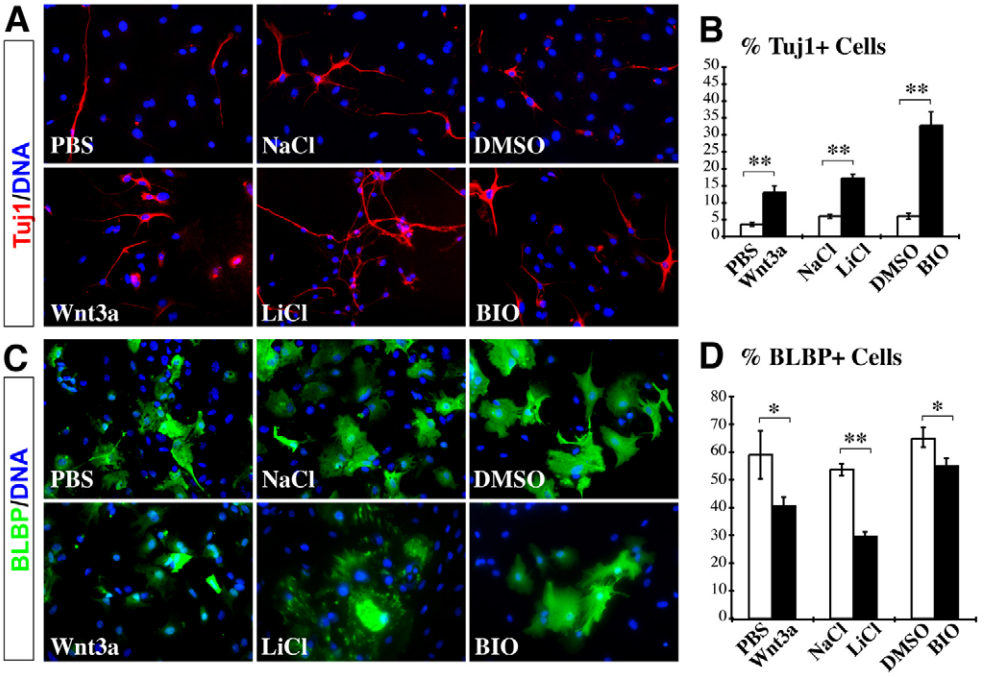

Fig. 6. Effects of Wnt stimulation on differentiation from dissociated OE sphere cells. (**A**,**B**) The percentage of Tuj1$^+$ OSNs was significantly increased by treatment with Wnt3a, LiCl and BIO compared with the controls. (**C**,**D**) The percentage of BLBP$^+$ glial lineage cells was decreased by treatment with Wnt signaling activators. *P<0.05; **P<0.01.

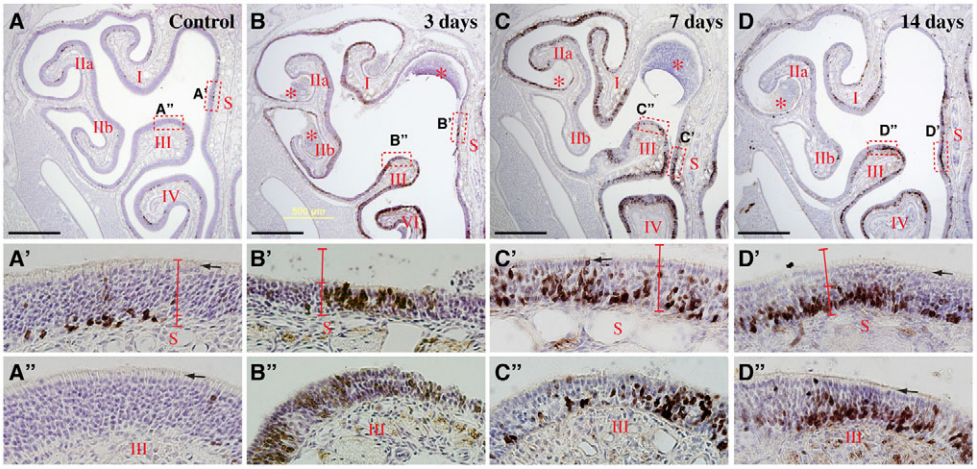

Fig. 7. Dramatic changes in the TOPeGFP$^+$ basal cells and OE morphology during forced OE regeneration in adult mice. (**A–A″**) Relatively low activity of TOPeGFP expression (immunolabeled with brown DAB products) in the control animal injected with saline. (**B–D″**) Robustly increased TOPeGFP expression during forced OE regeneration at 3 days (B-B″), 1 week (C-C″), and 2 weeks (D-D″) after intraperitoneal administration with methimazole. Dashed rectangles in A–D indicate respective enlarged regions in A′–D″. Asterisks in B–D indicate the degenerated OE-like tissue. Arrows in A′–D″ indicate the surface mucous layer. Red lines in A′–D′ indicate the thickness of the normal OE and the changes of the TOPeGFP$^+$ basal layers. I, superior nasal turbinate; IIa, IIb, branches of the middle turbinate; III, VI, inferior turbinates; S, septum. Scale bars: 500 µm.

compared with the control animals injected with the vehicle solution after methimazole treatment. These results suggest that the activation of canonical Wnt/β-catenin signaling is required for the active regeneration potential in the injured OE. We also tested the effect of the Wnt activator lithium on methimazole-induced regeneration. At 7 days post injury, we detected a 1.67-fold increase (with statistical significance) of Omp$^+$ OSNs, a 1.38-fold increase of TOPeGFP$^+$ basal cells, and a mild increase of the BrdU$^+$ OE progenitors in the LiCl-treated animals compared with the control (Fig. 8F,G,J and data not shown). At 3 days post injury, we detected a 1.78-fold increase (with statistical significance) of BrdU$^+$ cells in the septal OE of the LiCl-treated (compared with the NaCl-treated) animals after injury (Fig. 8H–J). These results suggest an early effect of the Wnt signaling activator in promoting proliferation of OE stem cells or progenitors for regeneration.

Discussion

Wnt signaling responsiveness monitored by transgenic reporters in early postnatal olfactory epithelium

Using our newly generated Wnt signaling reporter TOPeGFP transgenic mice, the current results demonstrate that TOPeGFP is

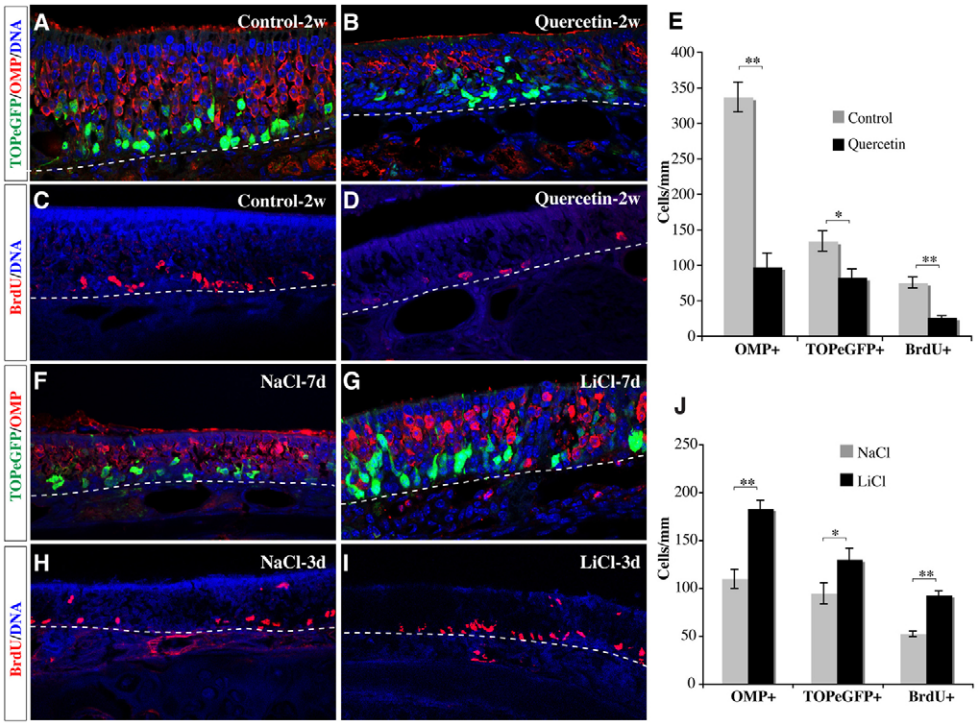

Fig. 8. In vivo administration of small molecules of Wnt signaling modulators altered OE regeneration in adult mice. (**A–E**) Intraperitoneal administration of a Wnt signaling inhibitor Quercetin significantly reduced the numbers of Omp$^+$ OSNs, BrdU$^+$ proliferating and TOPeGFP$^+$ basal cells in the septal OE after 2 weeks of methimazole treatment. (**F–J**) Intraperitoneal administration of the Wnt signaling activator lithium significantly increased the numbers of Omp$^+$ OSNs and TOPeGFP$^+$ basal cells at 7 days and BrdU$^+$ proliferating OE basal cells at 3 days, in the septal OE after methimazole treatment. Dashed lines indicate the basal membrane dividing OE and lamina propria. *P<0.05; **P<0.01.

activated in basal progenitor cells in the neonatal OE, suggesting an important role of the canonical Wnt/β-catenin signaling pathway in regulating neurogenesis and cell fate in the OE. Our findings here with TOPeGFP mice encompass the labeling observed by ourselves and other groups on the two previously available Wnt-signaling reporter strains, TOPgal and BATgal; all three reporter strains show Wnt activation in a unique subgroup of OECs in the olfactory bulb. Likewise, TOPeGFP shows strong labeling of basal cells in the OE that are weakly labeled or unlabeled by TOPgal or BATgal activity (Booker-Dwyer et al., 2008; Zaghetto et al., 2007). The differences between current and previous findings in the OE across the reporter strains are probably due to differences in the nature of the different reporter genes (eGFP vs β-galactosidase) and the different integration sites of the transgenic constructs in these mouse lines.

Wnt signaling components expressed in the developing OE

Consistent with the activation of Wnt signaling reporters in the early postnatal OE, the gene expression of multiple Wnts and Fzd receptors have been selectively determined in the developing OE of mice (Rodriguez-Gil and Greer, 2008; Wang et al., 2008; Zaghetto et al., 2007). Zaghetto and colleagues detected ten Wnts (including *Wnt2*, *Wnt2b*, *Wnt3*, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt6*, *Wnt7b*, *Wnt9a* and *Wnt11*) and two Fzd receptors (*Fzd3* and *Fzd4*) from E13.5 OE by RT-PCR or in situ hybridization (Zaghetto et al., 2007). We have previously detected *Wnt1*, *Wnt3* and *Wnt5a* in E16.5 OE by in situ hybridization (Wang et al., 2008). A more recent study confirmed, in part, the OE expression of these Wnt signaling components by RT-PCR or immunohistochemistry; Wnt2 and Wnt3 were not found, but several additional Wnt signaling components (including Fzd1, Fzd6, Fzd7, Fzd10, Frzb, Sfrp1 and Sfrp2) were detected in the OE of E13 and P4 mice (Rodriguez-Gil and Greer, 2008). In the current study, we demonstrate the expression of several Wnt signaling components in P5 OE and show that *Wnt2*, *Wnt3a*, *Fzd3*, *Fzd9*, *Tcf7* (previously *Tcf1*), *Tcf7l1* (previously *Tcf3*), *Tcf7l2* (previously *Tcf4*) and *Axin2* are expressed in OE basal cells or sphere-forming cells, which makes them potential candidates for the Wnt signaling components that activate TOPeGFP in the OE basal cells. Axin2 is a direct target and feedback gene in the canonical Wnt/β-catenin signaling pathway (Jho et al., 2002; Lustig et al., 2002; Yan et al., 2001). The colocalization of *Axin2* mRNA with TOPeGFP in basal cells demonstrates the activation of canonical Wnt signaling and its reporter TOPeGFP in the OE basal cells. In particular, the expression of *Wnt3a* and *Fzd9* throughout most of the OE at P5, suggests a key ligand–receptor pair of Wnt signaling activators in the basal cell progenitors. Of four Tcf/Lef transcription factors tested, we showed that *Tcf7l2* is expressed in the entire OE and most densely in the basal cells, suggesting that it is a downstream effecter of the Wnt/β-catenin signaling pathway in the OE basal cells. Interestingly, we found high expression of a secreted inhibitory ligand, *Sfrp1*, in the sustentacular cells and the lamina mucosa, but no or low expression in the basal cells, suggesting that a negative regulatory mechanism exists to restrict the activation of canonical Wnt signaling and its reporter TOPeGFP in the OE basal cells.

Canonical Wnt signaling is crucial for self-renewal of OE stem cells

On the basis of sphere formation by the OE in vitro and RT-PCR, the current study demonstrates the activation of Wnt/β-catenin signaling, as monitored by TOPeGFP, and the expression of several Wnt signaling components in the OE spheres. In contrast to the high efficiency of CNS neurosphere formation and expansion (Deleyrolle and Reynolds, 2009; Reynolds and Weiss, 1996), sphere formation from P5 OE occurs with a relatively low frequency in primary cultures, and the P5 spheres or colonies have a very limited capacity to be passaged (Carter et al., 2004; Murdoch and Roskams, 2007). In our case, using culture conditions identical to those for CNS neurospheres, primary spheres from P5 OE are capable of producing secondary spheres with somewhat better efficiency, such that primary or secondary sphere formation might be a measure of the self-renewal potential of OE stem cells. Importantly, we have demonstrated that activation of Wnt signaling can promote the formation of both primary and secondary OE spheres, whereas inhibition represses sphere formation, suggesting that canonical Wnt/β-catenin signaling has a primary role in OE progenitor cell expansion and/or self-renewal. More significantly, the current study demonstrates that FACS-isolated TOPeGFP^{-} OE cells are unable to form spheres, whereas the sorted TOPeGFP^{+} cells can, albeit with lower efficiency compared with the unsorted cells. Owing to the lack of specific basal OE markers, the current study could not directly compare the ability to form neural spheres between TOPeGFP^{+} and TOPeGFP^{-} basal cells. Nevertheless, our findings suggest that canonical Wnt/β-catenin signaling is required for OE sphere formation or OE stem cell expansion in a cell-autonomous manner, and that Wnts and/or other signaling molecules secreted by surrounding TOPeGFP^{-} cells enhance progenitor cell expansion and renewal.

Our proliferation and apoptosis assays showed that Wnt promoted the proliferation of Sox2^{+} dissociated OE sphere cells without influencing cell survival, further demonstrating the important role of canonical Wnts in promoting the proliferation or self-renewal of neural stem cells or progenitors. Activation of Wnt signaling that is restricted to Sox2^{+} OE basal cells suggests a close relationship between Sox2 and Wnt signaling. Indeed, it has been reported that Sox2 might be regulated by Wnt signaling in the *Xenopus* retina and that Wnt signaling and Sox2 are involved in the transition from proliferation to differentiation in neurogenesis of the *Xenopus* retina or adult hippocampal (Agathocleous et al., 2009; Kuwabara et al., 2009; Van Raay et al., 2005). However, it remains unknown whether similar mechanisms exist in the OE system.

The role of canonical Wnt signaling in OE neurogenesis during development and regeneration

The current study on the differentiation of adherent OE spheres demonstrates that Wnt-treated OE stem cells are able to differentiate into several neuronal and non-neuronal lineages including GBCs, OSNs, OECs and sustentacular cells. We showed that K5^{+} HBCs are often located near the center of the differentiated OE sphere, whereas GBC2^{+} cells are found peripheral to them. From the in situ study, we found that TOPeGFP is expressed in a subpopulation of Sox2^{+} or K5^{+} HBCs, but in all Sox2^{+} GBCs of P5 OE. These basal cells are widely recognized as encompassing the proximate OSN precursors, as well as multipotent progenitors, suggesting that Wnt signaling also directly promotes neurogenesis. Indeed, we observed that the percentage of TuJ1^{+} OSNs was significantly increased by Wnt activators; at the same time, the percentage of BLBP^{+} cells was significantly decreased. In support of this, Wnt signaling has been shown to promote neuronal differentiation in the forebrain through direct upregulation of Ngn1 (Hirabayashi et al., 2004), a neuronal differentiation transcription factor that is also

expressed in OE basal progenitors and participates in the initiation of OE neurogenesis (Cau et al., 2002). These data raise the possibility that Wnt signaling promotes neuronal differentiation at least partially by regulating its downstream target, Ngn1, in OE basal progenitors. The reciprocity between Tuj1$^+$ and BLBP$^+$ cells as a consequence of Wnt activation suggests a common lineage.

From our regeneration studies in adult OE, we found robust activation of the Wnt signaling reporter TOPeGFP in basal cells during the forced OE regeneration process. The dramatic increase of the TOPeGFP$^+$ basal cells within 3–7 days after injury indicate that canonical Wnt signaling is required for rapid expansion of the adult OE stem cells or progenitors towards adult neurogenesis and OE regeneration. Indeed, the selective small molecule Wnt inhibitor Quercetin significantly prevented the regeneration potential demonstrated by the decrease of the TOPeGFP$^+$ cells, proliferating basal progenitors and differentiated olfactory sensory neurons in the injured OE. Quercetin showed a maximal inhibitory effect on Omp expression, suggesting an accumulated consequence of the inhibited Wnt signaling activity and proliferation of OE progenitors during the entire 2 week regeneration period. However, we found an early effect of lithium in promoting Wnt signaling activity, progenitor proliferation and OE neurogenesis. Together with the findings for the role of Wnt signaling in other adult stem cell niches (Fuchs, 2009; Goessling et al., 2009; Li and Clevers, 2010; Lie et al., 2005; Nusse, 2008; Reya and Clevers, 2005), our results suggest that canonical Wnt/β-catenin signaling has a crucial role in adult OE stem cells and regeneration under both physiological and pathological conditions.

Materials and Methods

Transgenic mice

The Wnt signaling reporter TOPeGFP mouse line was generated as follows. The 792 bp eGFP (enhanced green fluorescence protein) cassette excised with *Bgl*II and *Not*I from the pEGFP-N1 (Clontech, Mountain View, CA) was cloned into the same sites of the TOPflash construct (Upstate, Temecular, CA) to replace the luciferase gene. A 3.5 kb fragment was excised at *Bgl*I sites of the transgenic construct, which contains two opposing sets of three copies of the TCF binding site upstream of the thymidine kinase (TK) minimal promoter and eGFP open reading frame (Fig. 1A). The transgenic DNA fragment was then purified and pronuclear microinjected into the FVB embryos. Mice were housed in the vivarium of the UC Davis Medical School (Sacramento, CA). PCR genotyping was performed with the genomic DNA from the tails of newborns using primers for eGFP. All animal experiments were carried out under protocols approved by UC Davis Animal Care and Use Committees and following NIH guidelines.

Immunohistochemistry and BrdU incorporation

The transgenic pups at postnatal day (P) 3 and P5 were perfused with 4% paraformaldehyde. After being embedded and frozen in O.C.T. compound, coronal sections of the head were cut at 10–14 μm with a cryostat (Leica CM 1950). After pre-incubation with a blocking solution of 10% lamb serum for 2 hours at room temperature, the sections were incubated with one or two of the following primary antibodies: rabbit anti-GFP (1:50, Chemicon), goat anti-GFP (1:300, Rockland), rabbit anti-β-galactosidase (βgal) (1:1000, Cappel), rabbit anti-K5 (1:250-500, Abcam), mouse anti-GBC2 IgM (1:100) and goat anti-Omp (1:1000-2000, Wako). Acute BrdU incorporation was carried out by intraperitoneal (i.p.) injection of BrdU (100 μg/g body weight) 1 to 2 hours before tissue harvest, as described previously (Song et al., 2009; Zhou et al., 2006; Zhou et al., 2008; Zhou et al., 2004b). Successive BrdU incorporation was carried out by four i.p. injections of BrdU by every 2 hours, for a total of 8 hours before tissue harvest. The cryostat sections were pretreated with 2 N HCl for 30 minutes at 37°C, blocked with 10% lamb serum, and incubated with mouse anti-BrdU (1:30, Iowa Hybridoma Bank). The following secondary antibodies were used as appropriate: Alexa Fluor 488 or Alexa Fluor 568 donkey anti-rabbit IgG, Alexa Fluor 568 donkey anti-goat IgG, Alexa Fluor 594 donkey anti-mouse IgM and Alexa Fluor 594 goat anti-mouse IgG (all from Molecular Probes, Eugene, OR). The sections were counterstained with DAPI (1 mg/ml) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for confocal microscopy with an Olympus DSU Spinning Disk confocal microscope (Olympus BX61) and a laser-scanning spectral confocal microscope system (Nikon Eclipse TE2000-E2).

To count cell numbers on immunolabeled sections, we adopted methods as described previously (Piper et al., 2010). Briefly, confocal images were taken from the septum of OE every five successive sections of each animal (n=3). The total numbers of TOPeGFP, Sox2, K5, BrdU, Dcx and Omp single-positive and double-positive cells were counted. Percentage colocalization was calculated by dividing the number of double-positive by single-positive cells in each image. Data represent pooled results from staining of three animals.

In situ hybridization

Nonradioactive in situ hybridization (ISH) was performed using digoxigenin-labeled riboprobes as described previously (Zhou et al., 2006; Zhou et al., 2004a; Zhou et al., 2004b). Except for the Wnt3a probe, which was prepared from an EST clone, all other probes were amplified by RT-PCR from mouse cDNAs with the following primers: *Axin2*, CTGCTGACTTAAGAGAGACCAAG, GAAAGTCCGGAAGAGGTATG; *Tcf7l2*, AACGGCGGTGGAGGAGATGAC, CGAACGAGCATCCTTGAGG; *Fzd9*, GGCATTGGCTACAACCTGAC, ACGTACTGGAACTTCTCGGG; *Wnt2*, CACCTGCTGCACATAAGTACAC, TCTAAGGTGTTGGCTGACAG; *Wnt7b*, GGAGTCGAGAGGCTGCCTTCAC, ACGGGCATCCACAAAGCGAC; *Sfrp1*, ATTGTCCCCAAGAAGAAGAAAC, AAAAGCCACAACTGTAATGG. After color development, the sections were counterstained with nuclear fast red (Sigma) for 5–10 minutes at room temperature, dehydrated, and mounted by coverslipping with mountant. Brightfield digital images were taken with an Olympus BX61 microscope.

Primary and secondary OE sphere culture and treatment

The OE sphere culture was carried out as previously described (Barraud et al., 2007) with minor modifications. Briefly, fresh olfactory mucosa from newborn mice (P3–P5) was dissected into ice-cold phosphate-buffered saline (PBS). Tissues were digested with 0.125% trypsin at 37°C for 15 minutes and quenched with 0.25 mg/ml trypsin inhibitor plus 20 μg/ml DNaseI for 5 minutes. After mechanical trituration, the cell suspension was filtered through a 40 μm mesh. Cells were then cultured in the Neurobasal medium (Gibco) with 2% B27, 1% N2, 20 ng/ml EGF, 20 ng/ml bFGF and 2 mM L-glutamine (all from Invitrogen) in low-attachment six-well plates (Nunc Brand). All cultures were maintained at 37°C with 5% CO_2 balanced air. The medium was half refreshed, and the growth factors were replenished every other day. For the primary sphere formation, cells were seeded at 2×10^4 cells/ml and the spheres were analyzed at 7 days in vitro (DIV). For secondary sphere formation, primary spheres were dissociated into a single-cell suspension, seeded at the same density, and cultured under the same conditions for 7 days before counting. Wnt3a (5 ng/ml, R&D), BIO (6-bromoindirubin-3′-oxime, 500 nM, Sigma), LiCl (2 mM Sigma), Dkk-1 (400 ng/ml, R&D), NaCl (2 mM, Sigma) and equal volume of DMSO (Sigma) or PBS controls were added in the culture medium and replenished every other day. The concentrations of these Wnt components/modulators were justified by the literature and our preliminary studies as described below. 1 ng/μl Wnt3a was used to promote the self-renewal of neurospheres derived from the neocortical subventricular zone (Kalani et al., 2008). We tested the effects of Wnt3a at different concentrations and found that it had limited effect at 1 ng/μl but promoted OE sphere formation at 5 ng/μl and 25 ng/μl with similar effects. Thus we used 5 ng/μl Wnt3a in the current study. 1 μM BIO was used to promote the proliferation of neonatal cardiomyocytes (Tseng et al., 2006). We tested various concentrations of BIO and determined a lower concentration of 500 nM for the current study. We determined a concentration of 2 mM LiCl for the in vitro study as referred to a proliferation study of cultured hippocampal progenitors (Wexler et al., 2008). We also determined a concentration of 400 ng/ml Dkk1 to inhibit Wnt signaling, as described in a previous study (Song et al., 2009).

Proliferation and apoptosis assays in dissociated OE sphere cells

Cultured OE spheres on DIV 7 with or without Wnt3a stimulation were seeded onto chamber slides (BD Biosciences) for attachment for 48 hours with the same culture condition. A final concentration of 10 μM BrdU was added and incubated for 4 hours, and then proceeded to BrdU immunolabeling. Rabbit anti-cleaved Caspase-3 (1:500, Cell signaling) was incubated with cells for apoptosis assays. In addition, TUNEL assay was conducted according to the manual of Deadend Flurometric TUNEL system (Promega).

Cell sorting

Fluorescence-activated cell sorting (FACS) was performed as previously described (Weeraratne et al., 2006) with minor modifications using a Cytomation Moflo cell sorter (Dako) and the Summit software package for data acquisition. Single-cell suspensions were generated from newborn olfactory mucosa as described above. After centrifugation at 189 ***g*** for 5 minutes, GFP-positive cells were sorted using FL1 channel. Wild-type pups from the same litter (i.e. without GFP reporter constructs) were used as the negative control for setting the gate. Tissue debris and dead cells were excluded by forward and side scatter. Sorted cells for qRT-PCR were collected in PBS. Sorted cells for culture were collected in Neurobasal medium containing 2% B27, 1% N2 and 2 mM L-glutamine. After sorting, cells were washed twice with Neurobasal medium containing B27 and N2, and then plated in the same condition for sphere assays.

Real-time quantitative RT-PCR and regular RT-PCR

For real-time RT-PCR, total RNA was extracted using Trizol agent from GFP^+ and GFP^- cells isolated by FACS from OE tissues collected from 7–10 TOPeGFP pups at P5. The RNA samples were treated with DNaseI to exclude the contamination of genomic DNA. After reverse transcription, semiquantitative PCR was carried out as described previously (Song et al., 2009; Song et al., 2010; Wang et al., 2011). The mRNA levels of *Axin2*, *Tcf7l2*, *Fzd9* and *Wnt3a* were normalized to the mRNA levels of the housekeeping gene *Gapdh* to allow comparisons among different experimental groups using the ΔC_t method. The following primer pairs were used for qPCR: *Gapdh*, TGCTGAGTATGTCGTGGAGTCT, CATATTTCTCGTGGTTCACACC; *Axin2*, AGTGAGACGCTCTCCCTCACCA, GAAACGGGCATAGGTTTGGTGGAC; *Tcf7l2*, GCACACATCGTTCAGAGCC, GGGTGTAGAAGTGCGGACA; *Fzd9*, CCAGTACGTGGAGAAGAGTC, GTGGAGAAGAAACACAACGC; *Wnt3a*, CTGAGCGACGGAGGGAGAAATG, CTCGGAATGAACCCTGCTCC; *GFP*, ATCTTCTTCAAGGACGACGGCAAC, TGTAGTTGTACTCCAGCTTGTGCC. For RT-PCR, primary OE spheres were collected by centrifugation at 7 DIV. 1 μg RNA was used for reverse transcription. PCR was done with the same primers used for preparing the various ISH probes. PCR was conducted with graded annealing temperature from 68°C to 60°C for 25 cycles. Performing PCR on the same amount of RNA without reverse transcription was the negative control.

Differentiation and immunocytochemistry

Investigation of neural differentiation was carried out as previously described (Barraud et al., 2007) with modifications. The primary spheres were mechanically dissociated at 7 DIV. Dissociated cells were plated in Neurobasal medium containing 1% fetal calf serum (FCS, Gibco), 1 μg/ml insulin, 20 μM dopamine, and 100 μM ascorbic acid (all from Sigma) (Murrell and Hunter, 1999) without EGF and FGF on the glass coverslips precoated with 25 mg/ml poly-D-lysine (Sigma) and 10 μg/ml mouse laminin (R&D system). Wnt3a, BIO, Dkk1, LiCl and NaCl were added at the same concentrations to the sphere cultures in the respective groups. Medium was half refreshed, and factors were replenished every other day. At 7 days of differentiation, cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and processed for immunocytochemistry. Both primary and secondary antibodies were used at one-half the concentration used for immunohistochemistry, except for the following antibodies: mouse anti-TuJ1 (1:300, Chemicon), rabbit anti-p75 (1:500, Chemicon), rabbit anti-BLBP (1:1500, Chemicon), mouse anti-Sus4 (1:100). The cells were incubated with secondary antibodies for 1 hour at room temperature.

In vitro cell counting and statistical analysis

The number of OE spheres in each well of the six-well plate was counted and divided by the number of plated cells (40,000 per well). The percentage of differentiated cells was determined by the number of the cells expressing TuJ1 (sensory neurons), BLBP (ensheathing cells and related glia), or p75 (ensheathing cells in ten random microscopic fields (at 100× magnification) divided by total number of DAPI-stained nuclei in the same fields. For each culture condition and for each marker, a minimum of 1000 cells was counted from three individual cultures prepared from separate dissections. Differences between culture conditions were assessed using one-way ANOVA followed by the Bonferroni's multiple comparison test. The s.e.m. values were considered significantly different when the value of the variance was $P<0.05$.

Adult OE regeneration and treatment

Adult TOPeGFP mice were injected intraperitoneally with 50 μg/g body weight of methimazole (Fluka 63760) in PBS on the first and the fourth day of treatment as reported (Bergman et al., 2002; Bergstrom et al., 2003; Brittebo, 1995; Genter et al., 1995). LiCl (Sigma, 254.34 mg/kg) (Greene et al., 2007; Ren et al., 2003), NaCl (Sigma, 351.1 mg/kg), quercetin (ACROS Organics, 100 mg/kg in Vehicle 1 containing 10% DMSO and 90% corn oil) (Schlachterman et al., 2008), XAV939 (Maybridge, 40 mg/kg in Vehicle 2 containing 10% DMSO, 10% ethanol, and 80% corn oil) (Huang et al., 2009), and their vehicles were injected i.p. once per day. At 3, 7 and 14 days after treatment, mice were injected with BrdU (i.p.) 1 hour before sampling. Immunohistochemistry of BrdU, Omp and GFP were performed as described above. Cell counting was done as described (Cheng and Reed, 2007) with minor modification. Briefly, GFP-, BrdU- and Omp-positive cells were counted under the microscope along OE lining the nasal septum from dorsal to ventral in three sections for each animal. OE length was determined by tracing the outline of the epithelium basal lamina using Slidebook software. The data is represented as mean ± s.e.m., and two-tailed Student's *t*-test was used for statistical analysis.

We thank Y. Li, E. McCauley, K. Wang, J. Chan, A. Wong, B. Palmer, J. Hui, and R. Huang for technical assistance; B. Gavino for advising on transgenic construction; UC Davis Mouse Biology Program for making the transgenic mice; B. McLaughlin, H. Shen, and M. Hoshi for help or advice on cell sorting; and D. Estrada for reading the manuscript. This work was supported by the Shriners Hospitals for Children (Research Grants 86100 and 87500 to C.J.Z.), NIH (1R01DE021696 to C.J.Z.), Children's Miracle Network (C.J.Z.), American Cancer Society (C.J.Z.), National Multiple Sclerosis Society (C.J.Z.), and UC Davis School of Medicine (Research Award to C.J.Z.). Y.-Z.W. is a postdoctoral fellow of the Shriners Hospitals for Children, and Q.G. is a postdoctoral fellow of the CIRM Stem Cell Training Program. Deposited in PMC for release after 12 months.

References

Agathocleous, M., Iordanova, I., Willardsen, M. I., Xue, X. Y., Vetter, M. L., Harris, W. A. and Moore, K. B. (2009). A directional Wnt/beta-catenin-Sox2-proneural pathway regulates the transition from proliferation to differentiation in the Xenopus retina. *Development* **136**, 3289-3299.

Bandyopadhyay, U., Biswas, K. and Banerjee, R. K. (2002). Extrathyroidal actions of antithyroid thionamides. *Toxicol. Lett.* **128**, 117-127.

Barraud, P., He, X., Zhao, C., Ibanez, C., Raha-Chowdhury, R., Caldwell, M. A. and Franklin, R. J. (2007). Contrasting effects of basic fibroblast growth factor and epidermal growth factor on mouse neonatal olfactory mucosa cells. *Eur. J. Neurosci.* **26**, 3345-3357.

Bergman, U., Ostergren, A., Gustafson, A. L. and Brittebo, B. (2002). Differential effects of olfactory toxicants on olfactory regeneration. *Arch. Toxicol.* **76**, 104-112.

Bergstrom, U., Giovanetti, A., Piras, E. and Brittebo, E. B. (2003). Methimazole-induced damage in the olfactory mucosa: effects on ultrastructure and glutathione levels. *Toxicol. Pathol.* **31**, 379-387.

Booker-Dwyer, T., Hirsh, S. and Zhao, H. (2008). A unique cell population in the mouse olfactory bulb displays nuclear beta-catenin signaling during development and olfactory sensory neuron regeneration. *Dev. Neurobiol.* **68**, 859-869.

Brittebo, E. B. (1995). Metabolism-dependent toxicity of methimazole in the olfactory nasal mucosa. *Pharmacol. Toxicol.* **76**, 76-79.

Calof, A. L., Bonnin, A., Crocker, C., Kawauchi, S., Murray, R. C., Shou, J. and Wu, H. H. (2002). Progenitor cells of the olfactory receptor neuron lineage. *Microsc. Res. Tech.* **58**, 176-188.

Carter, L. A., MacDonald, J. L. and Roskams, A. J. (2004). Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. *J. Neurosci.* **24**, 5670-5683.

Cau, E., Casarosa, S. and Guillemot, F. (2002). Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. *Development* **129**, 1871-1880.

Cavallaro, M., Mariani, J., Lancini, C., Latorre, E., Caccia, R., Gullo, F., Valotta, M., DeBiasi, S., Spinardi, L., Ronchi, A. et al. (2008). Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. *Development* **135**, 541-557.

Cheng, L. E. and Reed, R. R. (2007). Zfp423/OAZ participates in a developmental switch during olfactory neurogenesis. *Neuron* **54**, 547-557.

Chien, A. J., Conrad, W. H. and Moon, R. T. (2009). A Wnt survival guide: from flies to human disease. *J. Invest. Dermatol.* **129**, 1614-1627.

Ciani, L. and Salinas, P. C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat. Rev. Neurosci.* **6**, 351-362.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. *Cell* **127**, 469-480.

Deleyrolle, L. P. and Reynolds, B. A. (2009). Isolation, expansion, and differentiation of adult Mammalian neural stem and progenitor cells using the neurosphere assay. *Methods Mol. Biol.* **549**, 91-101.

Donner, A. L., Episkopou, V. and Maas, R. L. (2007). Sox2 and Pou2f1 interact to control lens and olfactory placode development. *Dev. Biol.* **303**, 784-799.

Fuchs, E. (2009). Finding one's niche in the skin. *Cell Stem Cell* **4**, 499-502.

Gelebart, P., Anand, M., Armanious, H., Peters, A. C., Dien Bard, J., Amin, H. M. and Lai, R. (2008). Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. *Blood* **112**, 5171-5179.

Genter, M. B., Deamer, N. J., Blake, B. L., Wesley, D. S. and Levi, P. E. (1995). Olfactory toxicity of methimazole: dose-response and structure-activity studies and characterization of flavin-containing monooxygenase activity in the Long-Evans rat olfactory mucosa. *Toxicol. Pathol.* **23**, 477-486.

Goessling, W., North, T. E., Loewer, S., Lord, A. M., Lee, S., Stoick-Cooper, C. L., Weidinger, G., Puder, M., Daley, G. Q., Moon, R. T. et al. (2009). Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* **136**, 1136-1147.

Greene, N. D., Bamidele, A., Choy, M., de Castro, S. C., Wait, R., Leung, K. Y., Begum, S., Gadian, D. G., Scott, R. C. and Lythgoe, M. F. (2007). Proteome changes associated with hippocampal MRI abnormalities in the lithium pilocarpine-induced model of convulsive status epilepticus. *Proteomics* **7**, 1336-1344.

Grigoryan, T., Wend, P., Klaus, A. and Birchmeier, W. (2008). Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev.* **22**, 2308-2341.

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. (2004). The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* **131**, 2791-2801.

Huang, S. M., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S. et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* **461**, 614-620.

Ille, F. and Sommer, L. (2005). Wnt signaling: multiple functions in neural development. *Cell. Mol. Life Sci.* **62**, 1100-1108.

Iwai, N., Zhou, Z., Roop, D. R. and Behringer, R. R. (2008). Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. *Stem Cells* **26**, 1298-1306.

Jagtap, S., Meganathan, K., Wagh, V., Winkler, J., Hescheler, J. and Sachinidis, A. (2009). Chemoprotective mechanism of the natural compounds, epigallocatechin-3-O-gallate, quercetin and curcumin against cancer and cardiovascular diseases. *Curr. Med. Chem.* **16**, 1451-1462.

Jang, W., Youngentob, S. L. and Schwob, J. E. (2003). Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. *J. Comp. Neurol.* **460**, 123-140.

Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* **22**, 1172-1183.

Kalani, M. Y., Cheshier, S. H., Cord, B. J., Bababeygy, S. R., Vogel, H., Weissman, I. L., Palmer, T. D. and Nusse, R. (2008). Wnt-mediated self-renewal of neural stem/progenitor cells. *Proc. Natl. Acad. Sci. USA* **105**, 16970-16975.

Kuwabara, T., Hsieh, J., Muotri, A., Yeo, G., Warashina, M., Lie, D. C., Moore, L., Nakashima, K., Asashima, M. and Gage, F. H. (2009). Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. *Nat. Neurosci.* **12**, 1097-1105.

Leung, C. T., Coulombe, P. A. and Reed, R. R. (2007). Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat. Neurosci.* **10**, 720-726.

Li, L. and Clevers, H. (2010). Coexistence of quiescent and active adult stem cells in mammals. *Science* **327**, 542-545.

Lie, D. C., Colamarino, S. A., Song, H. J., Desire, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. et al. (2005). Wnt signalling regulates adult hippocampal neurogenesis. *Nature* **437**, 1370-1375.

Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.

Loseva, E., Yuan, T. F. and Karnup, S. (2009). Neurogliogenesis in the mature olfactory system: a possible protective role against infection and toxic dust. *Brain Res. Rev.* **59**, 374-387.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P. M., Birchmeier, W. et al. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol. Cell. Biol.* **22**, 1184-1193.

Lyu, J., Yamamoto, V. and Lu, W. (2008). Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. *Dev. Cell* **15**, 773-780.

Michaelidis, T. M. and Lie, D. C. (2008). Wnt signaling and neural stem cells: caught in the Wnt web. *Cell Tissue Res.* **331**, 193-210.

Moon, R. T., Kohn, A. D., De Ferrari, G. V. and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* **5**, 691-701.

Murakami, A., Ashida, H. and Terao, J. (2008). Multitargeted cancer prevention by quercetin. *Cancer Lett.* **269**, 315-325.

Murdoch, B. and Roskams, A. J. (2007). Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. *J. Mol. Histol.* **38**, 581-599.

Muroyama, Y., Kondoh, H. and Takada, S. (2004). Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochem. Biophys. Res. Commun.* **313**, 915-921.

Murrell, J. R. and Hunter, D. D. (1999). An olfactory sensory neuron line, odora, properly targets olfactory proteins and responds to odorants. *J. Neurosci.* **19**, 8260-8270.

Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat. Neurosci.* **5**, 525-532.

Nusse, R. (2008). Wnt signaling and stem cell control. *Cell Res.* **18**, 523-527.

Park, C. H., Chang, J. Y., Hahm, E. R., Park, S., Kim, H. K. and Yang, C. H. (2005). Quercetin, a potent inhibitor against beta-catenin/Tcf signaling in SW480 colon cancer cells. *Biochem. Biophys. Res. Commun.* **328**, 227-234.

Piper, M., Barry, G., Hawkins, J., Mason, S., Lindwall, C., Little, E., Sarkar, A., Smith, A. G., Moldrich, R. X., Boyle, G. M. et al. (2010). NFIA controls telencephalic progenitor cell differentiation through repression of the Notch effector Hes1. *J. Neurosci.* **30**, 9127-9139.

Ren, M., Senatorov, V. V., Chen, R. W. and Chuang, D. M. (2003). Postinsult treatment with lithium reduces brain damage and facilitates neurological recovery in a rat ischemia/reperfusion model. *Proc. Natl. Acad. Sci. USA* **100**, 6210-6215.

Reya, T. and Clevers, H. (2005). Wnt signalling in stem cells and cancer. *Nature* **434**, 843-850.

Reynolds, B. A. and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**, 1-13.

Rodriguez-Gil, D. J. and Greer, C. A. (2008). Wnt/Frizzled family members mediate olfactory sensory neuron axon extension. *J. Comp. Neurol.* **511**, 301-317.

Roman-Gomez, J., Cordeu, L., Agirre, X., Jimenez-Velasco, A., San Jose-Eneriz, E., Garate, L., Calasanz, M. J., Heiniger, A., Torres, A. and Prosper, F. (2007). Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. *Blood* **109**, 3462-3469.

Sakamoto, T., Kondo, K., Kashio, A., Suzukawa, K. and Yamasoba, T. (2007). Methimazole-induced cell death in rat olfactory receptor neurons occurs via apoptosis triggered through mitochondrial cytochrome c-mediated caspase-3 activation pathway. *J. Neurosci. Res.* **85**, 548-557.

Schlachterman, A., Valle, F., Wall, K. M., Azios, N. G., Castillo, L., Morell, L., Washington, A. V., Cubano, L. A. and Dharmawardhane, S. F. (2008). Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model. *Transl. Oncol.* **1**, 19-27.

Schwob, J. E. (2002). Neural regeneration and the peripheral olfactory system. *Anat. Rec.* **269**, 33-49.

Sommer, L. (2004). Multiple roles of canonical Wnt signaling in cell cycle progression and cell lineage specification in neural development. *Cell Cycle* **3**, 701-703.

Song, L., Li, Y., Wang, K., Wang, Y. Z., Molotkov, A., Gao, L., Zhao, T., Yamagami, T., Wang, Y., Gan, Q. et al. (2009). Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. *Development* **136**, 3161-3171.

Song, L., Li, Y., Wang, K. and Zhou, C. J. (2010). Cardiac neural crest and outflow tract defects in Lrp6 mutant mice. *Dev. Dyn.* **239**, 200-210.

Tome, M., Lindsay, S. L., Riddell, J. S. and Barnett, S. C. (2009). Identification of nonepithelial multipotent cells in the embryonic olfactory mucosa. *Stem Cells* **27**, 2196-2208.

Tseng, A. S., Engel, F. B. and Keating, M. T. (2006). The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. *Chem. Biol.* **13**, 957-963.

Van Raay, T. J., Moore, K. B., Iordanova, I., Steele, M., Jamrich, M., Harris, W. A. and Vetter, M. L. (2005). Frizzled 5 signaling governs the neural potential of progenitors in the developing Xenopus retina. *Neuron* **46**, 23-36.

Wang, Y. Z., Molotkov, A., Song, L., Li, Y., Pleasure, D. E. and Zhou, C. J. (2008). Activation of the Wnt/beta-catenin signaling reporter in developing mouse olfactory nerve layer marks a specialized subgroup of olfactory ensheathing cells. *Dev. Dyn.* **237**, 3157-3168.

Wang, Y., Song, L. and Zhou, C. J. (2011). The canonical Wnt/β-catenin signaling pathway regulates Fgf signaling for early facial development. *Dev. Biol.* **349**, 250-260.

Weeraratne, S. D., Valentine, M., Cusick, M., Delay, R. and Van Houten, J. L. (2006). Plasma membrane calcium pumps in mouse olfactory sensory neurons. *Chem. Senses* **31**, 725-730.

Wexler, E. M., Geschwind, D. H. and Palmer, T. D. (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol. Psychiatry* **13**, 285-292.

Yan, D., Wiesmann, M., Rohan, M., Chan, V., Jefferson, A. B., Guo, L., Sakamoto, D., Caothien, R. H., Fuller, J. H., Reinhard, C. et al. (2001). Elevated expression of axin2 and hnkd mRNA provides evidence that Wnt/beta-catenin signaling is activated in human colon tumors. *Proc. Natl. Acad. Sci. USA* **98**, 14973-14978.

Zaghetto, A. A., Paina, S., Mantero, S., Platonova, N., Peretto, P., Bovetti, S., Puche, A., Piccolo, S. and Merlo, G. R. (2007). Activation of the Wnt-beta catenin pathway in a cell population on the surface of the forebrain is essential for the establishment of olfactory axon connections. *J. Neurosci.* **27**, 9757-9768.

Zhou, C. J., Pinson, K. I. and Pleasure, S. J. (2004a). Severe defects in dorsal thalamic development in low-density lipoprotein receptor-related protein-6 mutants. *J. Neurosci.* **24**, 7632-7639.

Zhou, C. J., Zhao, C. and Pleasure, S. J. (2004b). Wnt signaling mutants have decreased dentate granule cell production and radial glial scaffolding abnormalities. *J. Neurosci.* **24**, 121-126.

Zhou, C. J., Borello, U., Rubenstein, J. L. and Pleasure, S. J. (2006). Neuronal production and precursor proliferation defects in the neocortex of mice with loss of function in the canonical Wnt signaling pathway. *Neuroscience* **142**, 1119-1131.

Zhou, C. J., Molotkov, A., Song, L., Li, Y., Pleasure, D. E., Pleasure, S. J. and Wang, Y. Z. (2008). Ocular coloboma and dorsoventral neuroretinal patterning defects in Lrp6 mutant eyes. *Dev. Dyn.* **237**, 3681-3689.