

Expression of Gpr177, a Wnt Trafficking Regulator, in Mouse Embryogenesis

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Wls/Evi/Srt encoding a multipass transmembrane protein has been identified as a regulator for proper sorting and secretion of Wnt in flies. We have previously demonstrated that *Gpr177* is the mouse ortholog required for axis determination. *Gpr177* is a transcriptional target of Wnt that is activated to assist its subcellular distribution in a feedback regulatory loop. We, therefore, proposed that reciprocal regulation of Wnt and *Gpr177* is essential for the Wnt-dependent developmental and pathogenic processes. Here, we examine the expression pattern of *Gpr177* in mouse development. *Gpr177* is expressed in a variety of tissues and cell types during organogenesis. Furthermore, *Gpr177* is a glycoprotein primarily accumulating in the Golgi apparatus in signal-producing cells. The glycosylation of *Gpr177* is necessary for proper transportation in the secretory pathway. Our findings suggest that the *Gpr177*-mediated regulation of Wnt is crucial for organogenesis in health and disease. *Developmental Dynamics* 239:2102–2109, 2010. © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Members of the Wnt family trigger cellular signals essential for proper development of organisms (Logan and Nusse, 2004; Clevers, 2006). Aberrant regulation of an evolutionary conserved Wnt signal transduction pathway has been implicated in a variety of cancers and congenital diseases (van Amerongen and Berns, 2006; Grigoryan et al., 2008). Wnt signaling has been repetitively proven to be critical for human health and disease. Compared with the enormous wealth of knowledge on the events in signal-receiving cells, we know relatively little about the processes associated with

Wnt maturation, sorting, and secretion in signal-producing cells (Willert et al., 2003; Takada et al., 2006; Couandre and Korswagen, 2007; Hausmann et al., 2007). At the primary sequence level, Wnt proteins share a nearly invariant pattern of 23 Cys residues, an N-terminal signal sequence and several potential N-glycosylation sites (Miller, 2002). Although the role of glycosylation in Wnt secretion and function is not fully understood (Tanaka et al., 2002; Vincent and Dubois, 2002; Eaton, 2006), two lipid modifications present on the mature Wnt seem to be required for correct intracellular targeting of Wnt and signaling activity of the secreted protein

(Willert et al., 2003; Zhai et al., 2004; Takada et al., 2006). Based on recent studies in the fly, Wnt proteins might be secreted bound to lipoprotein particles (Panakova et al., 2005; Eaton, 2006). Alternatively, Wnt proteins might form high order complexes, thereby overcoming their hydrophobic property and potentiating their signaling capacity (Miller, 2002).

The mechanism underlying the sorting and secretion of Wnt remains largely elusive. Studies in the fly suggest that Wnt accumulates in cells where nonconventional secretory routes may be used (Strigini and Cohen, 2000; Marois et al., 2006). A significant fraction of Wnt is present in endocytic

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vesicles which retrieve back to the surface (Pfeiffer et al., 2002). The function of this retrieval is not clear, but it might allow Wnt to gain access to cellular compartments from which standard secretory molecules are excluded. Alternatively, Wnt might directly enter these vesicles during the secretion process as the fusion of secretory vesicles from the Golgi with endosomes has been reported (Futter et al., 1995; Ang et al., 2004). The identification of Wntless (Wls/Evi/Srt; Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006) has shown that this multipass transmembrane protein is a transporting regulator for Wnt production in *Drosophila*. Given the extensive Wnt family in higher organisms, the essential role of Wls in production of all Wnt proteins remains to be determined.

We have previously demonstrated that *Gpr177* is the mouse ortholog of *Drosophila* Wls, required for Wnt-mediated embryonic axis determination (Fu et al., 2009). In the *Gpr177*-deficient mutant, Wnt production is impaired, leading to disruption of Wnt signaling in the establishment of the anterior–posterior axis. As a Wnt direct target, *Gpr177* is activated by β -catenin and LEF/Tcf-dependent transcription. This activation then modulates Wnt production in a positive feedback loop. Our study has led to a hypothesis in which reciprocal regulation of Wnt and *Gpr177* is essential for normal developmental processes. Alterations of this regulatory circuit are causally linked to pathogenesis in human diseases.

In this study, we have examined the expression pattern of mouse *Gpr177* during development of various organs. This comprehensive survey reveals that *Gpr177* may modulate the Wnt pathway in a variety of tissues and cell types. Furthermore, *Gpr177* is a glycoprotein predominantly localized in the Golgi apparatus. Disturbance of N-linked glycosylation prevents the Golgi accumulation of *Gpr177*. Given the established role of Wnt signaling in health and disease (<http://www.stanford.edu/~rnusse/wntwindow.html>), our findings suggest that the *Gpr177*-mediated Wnt regulation is critical for organogenesis, including neural development, craniofacial morphogenesis, and the other developmental and pathogenic processes, especially

related to epithelial–mesenchymal interaction.

RESULTS AND DISCUSSION

Expression Pattern of *Gpr177* mRNA and Protein in Mouse Embryogenesis

To study the potential involvement of *Gpr177* during mouse embryogenesis, we analyzed its expression by in situ hybridization. A majority of organs began to form in the developing mouse embryo after 13.5 days post coitum (dpc). We detected the *Gpr177* transcript in various neural tissues, craniofacial prominences, developing skeleton, and several internal organs (Fig. 1A,B). Strong expression of *Gpr177* was shown in certain regions of the brain, which include forebrain, midbrain, hindbrain, and Rathke's pouch (future pituitary gland). Craniofacial tissues, such as tooth primordium, tongue, olfactory epithelium, Meckel's cartilage, inner ear, esophagus, and lip, were also positive for the staining. In the trunk region, the stained signals were present in dorsal root ganglia, spinal cord, and cartilage primordium. The *Gpr177* transcript could also be found in lung, kidney, intestine, thymic primordium, and urethra.

We next investigated the presence of *Gpr177* protein during embryogenesis using an antibody, which we developed previously against the carboxyl terminus (Fu et al., 2009). In our previous report, this antibody recognized a ~60-Kd protein, which is absent in the *Gpr177* mutants, suggesting the specificity of this antibody (Fu et al., 2009). In addition, immunostaining was able to detect a strong presence of *Gpr177* in the control mesoderm at embryonic day (E) 7.5, while the *Gpr177*-positive mesoderm was absent in the mutant (Fu et al., 2009). The protein expression analysis was in agreement with the in situ hybridization study (Fig. 1C,D). Strongest levels of *Gpr177* were identified in the brain regions with a very restricted expression pattern around hippocampus, thalamus, hypothalamus, and ventricles. Similar to the in situ expression study, Rathke's pouch, dorsal root ganglia, spinal cord, cartilage primordium, lung, kidney, intestine, thymic primordium, ovary, and urethra were positive

for the staining. Immunostained signals were also observed in the craniofacial regions, including tooth primordium, tongue, olfactory epithelium, Meckel's cartilage, inner ear, esophagus, and lip. The protein expression pattern coincides with that of the *Gpr177* transcript, indicating the specificity of our *Gpr177* antibody.

Early Neural Development

Closer examination of *Gpr177* in neural development found that it is highly expressed in the neural epithelial cells of various tissues (Fig. 2). Most prominent expression was detected in the dentate gyrate epithelium of hippocampus (Fig. 2A,E,I,M) and epithelium of the thalamus surrounding the third ventricle (Fig. 2I,M). The *Gpr177* expression was distinguished in the neural epithelial cells of midbrain (Fig. 2B,F,J,N), cerebellum (Fig. 2C,G,K,O), pons (Fig. 2D,H), medulla (Fig. 2D,K,O), and spinal cord (Fig. 2L,P) that line the aqueduct, fourth ventricle, and central canal, especially in the ventral and dorsal regions. The choroid epithelial cells of the choroid plexus in the lateral ventricle (Fig. 2I,M) and fourth ventricle (Fig. 2C,G,K,O) also exhibited the *Gpr177* transcript and protein at high levels. The expression in the proliferating zones of these tissues suggests that *Gpr177* may have a role in the expansion of neural stem/progenitor cells. Mouse genetic analyses have demonstrated that members of the Wnt family are essential for early neural development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Hall et al., 2000; Lee et al., 2000). In addition, several Wnt signaling molecules, e.g., GSK3 and Axin1, are essential for development of neuroectoderm (Zeng et al., 1997; Kim et al., 2009). The deletion of Axin1 has been reported in human medulloblastoma (Dahmen et al., 2001). Therefore, it is highly possible that the *Gpr177*-mediated Wnt production is critically linked to neural development in health and disease.

Craniofacial Morphogenesis

During craniofacial morphogenesis, the expression of *Gpr177* was detected in many cell types and tissues (Fig. 3). In the developing incisors and molars,

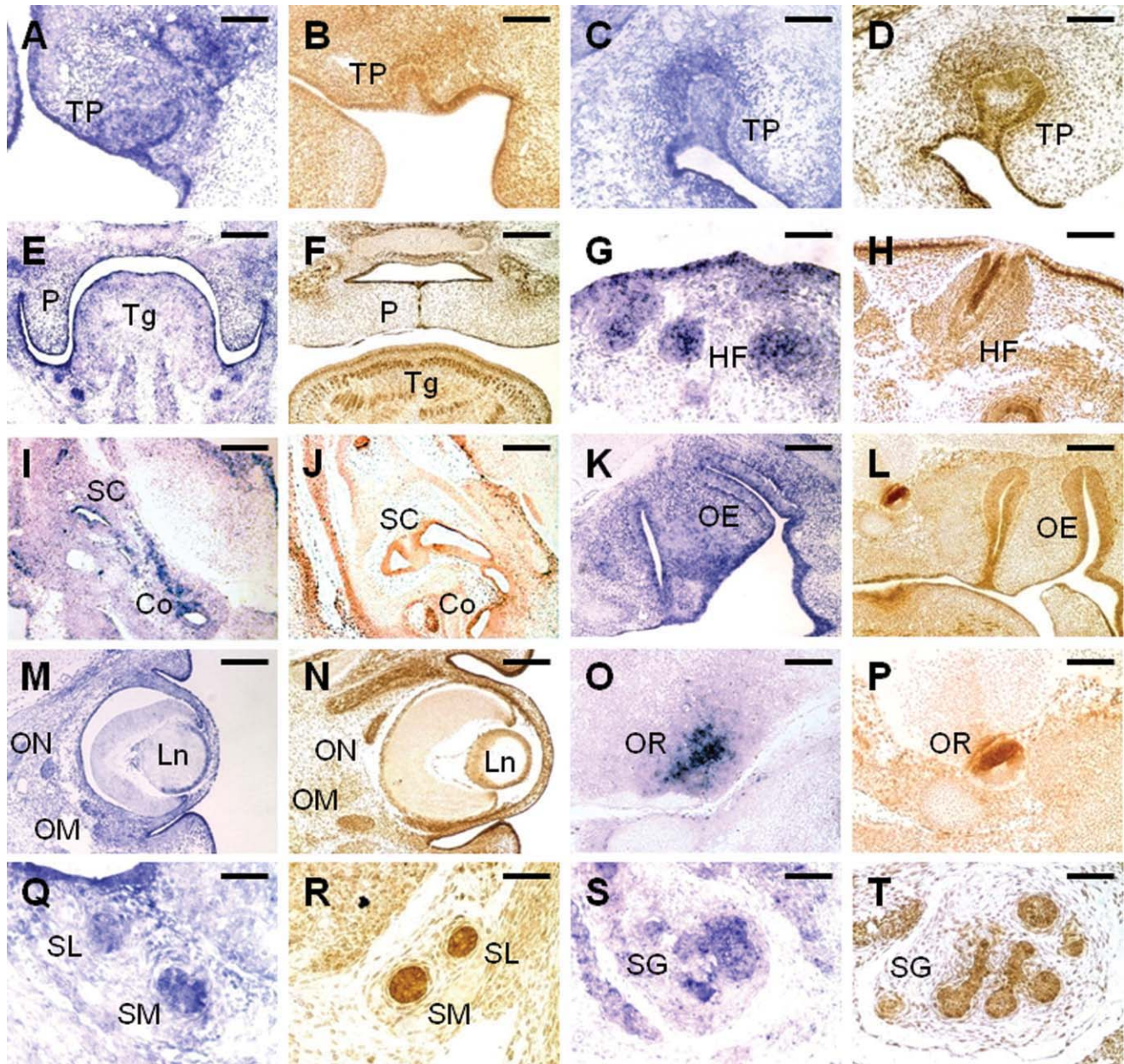


Fig. 3. Expression of *Gpr177* during craniofacial morphogenesis. **A–T:** In situ hybridization (**A,C,E,G,I,K,M,O,Q,S**) and immunostaining (**B,D,F,H,J,L,N,P,R,T**) analyses reveal that a variety of craniofacial tissues and cell types express *Gpr177* at embryonic day (E) 14.5. Co, cochlea; HF, hair follicle; Ln, lens; OE, olfactory epithelium; OM, ocular muscle; ON, optic nerve; OR, optic recess; P, palate; SC, semicircular canal; SG, salivary gland; SL, sublingual duct; SM, submandibular duct; Tg, tongue; TP, tooth primordium. Scale bars = 100 μm in **A–D,G,H,I,S,T**, 50 μm in **E,F**, 200 μm in **J,L–P**.

expression was also observed, suggesting that *Gpr177* may regulate the sorting and secretion of *Wnt4*, *Wnt7b*, *Wnt9b*, and *Wnt11*, which have been implicated in kidney organogenesis (Stark et al., 1994; Majumdar et al., 2003; Park et al., 2007; Karner et al., 2009; Yu et al., 2009). In agreement with mouse genetic studies of the *Wnt* family proteins (Li et al., 2002; Shu et al., 2002; Rajagopal et al., 2008; Goss et al., 2009), our data showed

that *Gpr177* is expressed in both epithelial and mesenchymal cells, potentially involved in lung development (Fig. 4E,F). A selective expression of *Gpr177* was found in the thymic primordium (Fig. 4G,H). Given the well-established role of *Wnt* in hematopoiesis (Staal and Clevers, 2005; Grigoryan et al., 2008; Malhotra and Kincaid, 2009), *Gpr177* is likely to play a role in development of the hematopoietic lineages. In the developing gut,

the expression was found in both epithelium and mesenchyme of the esophagus (Fig. 4I,J), mid gut (Fig. 1B), and duodenum (Fig. 4K,L), suggesting that the *Gpr177*-dependent regulation of *Wnt* may be critical for development of the digestive system.

Glycosylation of *Gpr177*

We previously showed that *Gpr177* is differentially localized in the *Wnt*-

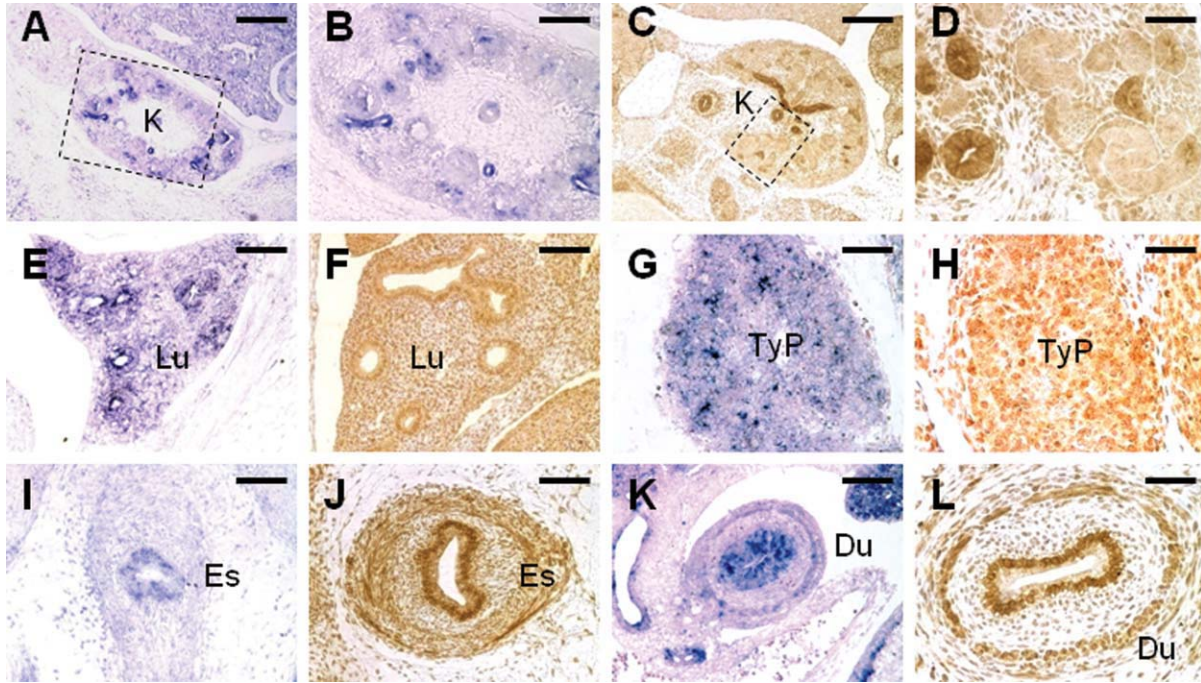


Fig. 4. Expression of Gpr177 in development of multiple organs. **A–L:** The mRNA and protein expression of Gpr177 is examined by in situ hybridization (**A,B,E,G,I,K**) and immunostaining (**C,D,F,H,J,L**) of embryonic day (E) 14.5 embryos, respectively. Insets in **A, C** denote higher magnification in **B, D**, respectively. Du, duodenum; Es, esophagus; K, kidney; Lu, lung; TyP, thymic primordium. Scale bars = 200 μm in **A,C**, 100 μm in **B,E,F,I,K**, 50 μm in **D,G,H,J,L**.

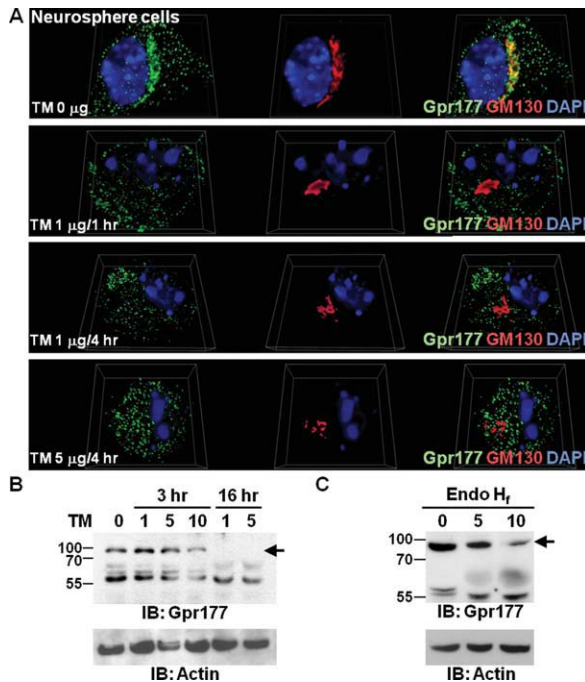


Fig. 5. Glycosylation is required for Golgi distributions of Gpr177. **A:** Neurosphere cells were treated with Tunicamycin (TM) for different concentrations and time periods as indicated. Three-dimensional (3D) imaging of the immunostained cells shows the localization of endogenous Gpr177 (green) and GM130 (red), counterstained with DAPI (4',6-diamidino-2-phenylidole-dihydrochloride; blue). Note that the TM treatment disrupts the colocalization of Gpr177 and GM130 in Golgi. **B:** Immunoblot analysis of Gpr177 reveals that TM abolishes the detection of Gpr177 with slow mobility (arrow) in neural stem cells. The number indicates the drug concentration ($\mu\text{g/ml}$) present in culture media. The expression level of Actin was analyzed as a loading control. **C:** Immunoblot analysis shows that the slow migrating band (arrow) detected by the Gpr177 antibody disappears after the addition of Endo H_f. The number presents the amount of enzyme (μl : 1,000 units per μl) added to the in vitro assays. The expression level of Actin was analyzed as a loading control.

producing and non-Wnt-producing cells (Fu et al., 2009). The differential compartmentalization is dependent upon the positive feedback regulation of Wnt to activate the Gpr177 expression at the transcriptional level (Fu et al., 2009). The Golgi accumulation of Gpr177 can only be identified in the signal-producing cells, such as neural stem cells (Fu et al., 2009). Because membrane and secreted proteins are modified by saccharides, we examined whether inhibition of glycosylation interferes with the Golgi accumulation of Gpr177. In the culture of neurosphere cells, Gpr177 is colocalized with a Golgi marker GM130 (Fig. 5A). The addition of tunicamycin, which inhibits the enzyme GlcNAc phosphotransferase involved in the first step of glycoprotein synthesis, affects concentrations of Gpr177 in the Golgi apparatus (Fig. 5A). Treatment of tunicamycin prevented the colocalization of Gpr177 with GM130, suggesting that glycosylation is required for the Golgi accumulation. Immunoblot analysis indicated that a slow migrating band disappears after the tunicamycin treatment (Fig. 5B). Furthermore, the addition of Endo H_f diminished the detection of Gpr177 with slow mobility (Fig. 5C). The results suggest that

Gpr177 is modified by N-linked glycosylation required for proper sorting within the cells.

In summary, we have performed a comprehensive survey on the expression of Gpr177 using in situ hybridization and immunostaining analyses. The expression patterns of mRNA and protein are in agreement with each other, supporting the notion that Wnt signaling is critical for development of multiple organs. Our prior discovery that Gpr177 is essential for modulating canonical Wnt in early embryogenesis suggests that their reciprocal regulations are likely to be important for other developmental processes. Gpr177 might also be associated with the Wnt-mediated malignant transformation of these organs. The expression of Gpr177 in development of several organs requiring the epithelial–mesenchymal interaction adds another layer of regulation for signal-producing and signal-receiving cells. In addition, the Wnt-producing cells are able to initiate paracrine as well as autocrine signaling effects. Gpr177 seems to be expressed in places where noncanonical Wnt proteins have essential functions. However, whether noncanonical Wnt is regulated by Gpr177 remains an important question to be determined, especially to be assessed functionally by genetic analysis. Using large-scale meta-analysis of genome-wide association, a recent study has identified *GPR177* as 1 of the 20 bone-mineral-density loci in humans (Rivadeneira et al., 2009). Among them, *CTNNB1*, encoding β -catenin, is another locus identified at the genome-wide study level. The finding suggests that Gpr177 and β -catenin are master regulators for Wnt production and signaling, respectively. One can expect further discovery revealing the significance of the Gpr177-mediated regulation of Wnt in skeletal development and disease.

EXPERIMENTAL PROCEDURES

Mouse embryos were first fixed, paraffin embedded, sectioned, and stained with hematoxylin/eosin for histological evaluation as described (Yu et al., 2007; Hsu et al., 2010). Sections were then subject to in situ hybridization with

digoxigenin-labeled antisense RNA probes or immunological staining with avidin:biotinylated enzyme complex as described (Yu et al., 2005a; Liu et al., 2007; Chiu et al., 2008). For in situ hybridization, RNA probes for detecting the Gpr177 transcript as well as the detailed method were described previously (Chiu et al., 2008; Fu et al., 2009). Rabbit polyclonal antibodies were generated for the immunostaining analysis of Gpr177 as described (Fu et al., 2009). Briefly, sections were deparaffinized and hydrated, followed by antigen unmasking (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide. After addition of primary antibodies, sections were incubated with horseradish peroxidase-conjugated secondary antibodies. The staining was then visualized by enzymatic color reaction according to the manufacture's specification (Vector Laboratories). Images were analyzed using Zeiss AXIO OBSERVER with AXIOCAM or Nikon SMZ-1500 with SPOT-RT microscope imaging systems (Yu et al., 2005b; Chiu et al., 2008; Liu et al., 2008).

Isolation and culture of primary neurospheres were performed as described (Fu et al., 2009). Briefly, telencephalons of E12.5 mouse embryos were recovered and mechanically dissociated in DMEM/F12 medium (1:1; Invitrogen, Carlsbad, CA), followed by filtering through a 70- μ m nylon mesh (BD Biosciences, Bedford, MA). Cells were then cultured in DMEM/F12 medium, supplemented with Insulin-Transferin-Selenium supplements (Invitrogen), epithelial growth factor (20 ng/ml; Sigma-Aldrich, St. Louis, MO), and antibiotic solution (Sigma-Aldrich), at 37°C in a humidified 5% CO₂ incubator. After 5 days, neurospheres were harvested by centrifugation, dissociated by Trypsin-ethylenediaminetetraacetic acid (Sigma-Aldrich), and continued to be cultured for next passages or used in experimental procedures.

Immunostaining of cultured neurosphere cells was performed using indirect fluorescent staining techniques described previously (Fu et al., 2009). Images were taken using AXIO OBSERVER with AXIOCAM microscope imaging system, followed by deconvolution, three-dimensional imaging analysis. Immunoblot analysis was

performed as described (Liu et al., 2008; Fu et al., 2009). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemical luminescence-mediated visualization (GE Healthcare, Piscataway, NJ) and autoradiography.

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