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Expression of Gpr177, a Wnt trafficking regulator, in mouse embryogenesis

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Abstract

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 orthologue required for axis determination. *Gpr177* is a transcriptional target of Wnt which 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.

Keywords

Gpr177; Wntless; Evi; Sprinter; β -catenin; Wnt production; Wnt signaling; organogenesis

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 to 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; Coudreuse 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).

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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 utilized (Strigini and Cohen, 2000; Marois et al., 2006). A significant fraction of Wnt is present in endocytic 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 orthologue 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 imply 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

The 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 dpc (days post coitum). We detected the *Gpr177* transcript in various neural tissues, craniofacial prominences, developing skeleton and several internal organs (Figure 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 investigate 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

E7.5 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 (Figure 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 (Figure 2). Most prominent expression was detected in the dentate gyrus epithelium of hippocampus (Figure 2A, E, I, M) and epithelium of the thalamus surrounding the third ventricle (Figure 2I, M). The Gpr177 expression was distinguished in the neural epithelial cells of midbrain (Figure 2B, F, J, N), cerebellum (Figure 2C, G, K, O), pons (Figure 2D, H), medulla (Figure 2D, K, O), and spinal cord (Figure 2L, P) that line the aqueduct, forth ventricle, and central canal, especially in the ventral and dorsal regions. The choroid epithelial cells of the choroid plexus in the lateral ventricle (Figure 2I, M) and forth ventricle (Figure 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 possibly 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 (Figure 3). In the developing incisors and molars, both epithelial cells and surrounding mesenchymal cells show expression of Gpr177 (Figure 3A–D). In humans, odonto-oncho-dermal dysplasia, a rare autosomal recessive inherited form of ectodermal dysplasia, was recently shown to be caused by a nonsense WNT10A mutation (Adaimy et al., 2007). The mutation of *AXIN2*, a gene encoding a negative regulator of Wnt essential for targeting β -catenin degradation, was linked to familial tooth agenesis (Lammi et al., 2004). Many previous studies (Jarvinen et al., 2006; Wang et al., 2009; Cobourne and Sharpe, 2010; Rooker et al., 2010) also support a potential role of Gpr177 in mediating Wnt production during tooth development.

Non-syndromic cleft lip with or without cleft palate is one of the most common birth defects. Genetic variations in several Wnt family members were shown to be associated with this phenotype in humans (Chiquet et al., 2008). Mouse genetic analysis also indicated that Wnt5a (He et al., 2008) and Wnt9b (Juriloff et al., 2006) are essential for palate development. Indeed, we were able to identify the presence of Gpr177 mRNA and protein during embryonic development of the lip (Figure 1A, C) and palate (Figure 3E, F). Strong Gpr177 expression was also found in the tongue (Figure 3E, F) that maybe related to the role of Wnt signaling in formation of taste papilla (Iwatsuki et al., 2007).

The Wnt pathway has a well established role in development of the skin and hair follicles (Alonso and Fuchs, 2003; Haegbarth and Clevers, 2009). There was no surprise to identify the Gpr177 transcript and protein in these regions (Figure 3G, H). Other sensory organs, including inner ear (Figure 3I, J), olfactory epithelium (Figure 3K, L), eye (Figure 3M–P), were also positive for the expression of Gpr177. Both canonical and non canonical Wnt proteins were shown to control formation of the inner ear (Riccomagno et al., 2005; Qian et al., 2007). It has been suggested that Wnt3 acts as an axon guidance molecule to mediate medial-lateral retinotectal topography (Schmitt et al., 2006). The localization of the Gpr177 mRNA and protein supports the role of Wnt signaling in retinal and lens development (Van Raay and Vetter, 2004; Lovicu and McAvoy, 2005; Silver and Rebay, 2005). However, the important function of Gpr177 and Wnt-mediated development in the olfactory system remains largely to be explored. In the salivary gland, Wnt stimulation was linked to pleomorphic adenoma, the most common type of salivary gland tumor in humans (Zhao et al., 2006; Declercq et al., 2008). Strong expression of Gpr177 was detected in the epithelial components of major salivary glands to a lesser extent in the mesenchymal regions (Figure 3Q–T). Nevertheless, the requirement of Wnt signaling in normal development of the salivary glands remains to be determined.

Internal organs

We next examined if Gpr177 is also expressed in development of other major organs. In the kidney, the Gpr177 mRNA and protein were detected predominantly in the ureteric epithelium, but not the differentiated comma and S-shape bodies (Figure 4A–D). A weak mesenchymal 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 (Figure 4E, F). A selective expression of Gpr177 was found in the thymic primordium (Figure 4G, H). Given the well established role of Wnt in hematopoiesis (Staal and Clevers, 2005; Grigoryan et al., 2008; Malhotra and Kincade, 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 (Figure 4I, J), mid gut (Figure 1B), duodenum (Figure 4K, L), suggesting 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-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 co-localized with a Golgi marker GM130 (Figure 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 (Figure 5A). Treatment of tunicamycin prevented the co-localization 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 (Figure 5B). Furthermore, the addition of Endo H_f diminished the detection of

Gpr177 with slow mobility (Figure 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 of Gpr177 essential for modulating canonical Wnt in early embryogenesis implies 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 non canonical Wnt proteins have essential functions. However, whether non canonical 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 one of the twenty 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 implies 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.

Methods

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 anti-sense 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-Transferrin-Selenium supplements (Invitrogen), EGF (20ng/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-EDTA (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, 3D 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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Figure 1.

Gpr177 transcript and protein are detected in multiple tissues and organs during mouse embryogenesis. In situ hybridization (A, B) and immunostaining (C, D) analyses show the expression pattern of Gpr177 in E14.5 and E13.5 embryos, respectively. Cb, cerebellum; ChP, choroid plexus; CC, cerebral cortex; CP, cartilage primordium; ccSC, central canal of spinal cord; DRG, dorsal root ganglia; Es, esophagus; Hc, hippocampus; Ht, hypothalamus; HB, hyoid bone; IE, inner ear; K, kidney; Lp, lip; Lu, lung; Mb, midbrain; MG, mid gut; MC, Meckel's cartilage; OE, olfactory epithelium; Ov, ovary; PP, pancreatic primordium; RP, Rathke's pouch; Tg, tongue; Th, thalamus; TP, tooth primordium; TyP, thymic primordium; Ur, urethra. Scale bar, 1 mm.

Figure 2.

Gpr177 gene is active in neural development. The expression pattern of *Gpr177* in various neural tissues and cell types of the developing embryos is analyzed by in situ hybridization (A–D, I–L; E14.5) and immunostaining (E–H; E13.5, M–P; E14.5). Cb, cerebellum; ChP, choroid plexus; CC, cerebral cortex; Hc, hippocampus; Ht, hypothalamus; Mb, midbrain; Me, medulla; SC, spinal cord; Th, thalamus. Scale bar, 200 μ m.

Figure 3.

Expression of *Gpr177* during craniofacial morphogenesis. 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 E14.5. 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 μ m (A–D, G, H, S, T); 50 μ m (Q, R); 200 μ m (E, F, I–P).

Figure 4.

Expression of Gpr177 in development of multiple organs. 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 E14.5 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 (A, C); 100 μm (B, E, F, I, K); 50 μm (D, G, H, J, L).

Figure 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. 3D imaging of the immunostained cells shows the localization of endogenous Gpr177 (green) and GM130 (red), counterstained with DAPI (blue). Note that the TM treatment disrupts the co-localization 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.