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RESEARCH ARTICLE

Gpr177/mouse Wntless Is Essential for Wnt-Mediated Craniofacial and Brain Development

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We have previously demonstrated that *Gpr177*, the mouse orthologue of *Drosophila Wls/Evi/Srt*, is required for establishment of the anterior–posterior axis. The *Gpr177* null phenotype is highly reminiscent to the loss of Wnt3, the earliest abnormality among all Wnt knockouts in mice. The expression of *Gpr177* in various cell types and tissues lead us to hypothesize that reciprocal regulation of Wnt and Gpr177 is essential for the Wnt-dependent developmental and pathogenic processes. Here, we create a new mouse strain permitting conditional inactivation of *Gpr177*. The loss of Gpr177 in the Wnt1-expressing cells causes mid/hindbrain and craniofacial defects which are far more severe than the Wnt1 knockout, but resemble the double knockout of Wnt1 and Wnt3a as well as β-catenin deletion in the Wnt1-expressing cells. Our findings demonstrate the importance of Gpr177 in Wnt1-mediated development of the mouse embryo, suggesting an overlapping function of Wnt family members in the Wnt1-expressing cells. *Developmental Dynamics 240:365–371, 2011.* © 2011 Wiley-Liss, Inc.

Key words: Gpr177; Wntless; Evi; Sprinter; **β**-catenin; neural crest; Wnt production; Wnt signaling

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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 this evolutionary conserved Wnt signal transduction pathway has been linked to a variety of cancers and congenital diseases (van Amerongen and Berns, 2006; Grigoryan et al., 2008). There is no question that Wnt signaling is intimately involved in human health and disease. While an enormous wealth of knowledge on the events occurring in signal-receiving cells has been obtained, the processes associated with Wnt maturation, sorting, and secretion in signal-producing cells remain largely elusive (Willert et al., 2003; Takada et al., 2006; Coudreuse and Korswagen, 2007; Hausmann et al., 2007).

We have recently identified *Gpr177*, the mouse orthologue of *Drosophila Wls/Evi/Srt*, encoding a multipass transmembrane protein essential for proper sorting and secretion of Wnt (Fu et al., 2009). Inactivation of *Gpr177* impairs patterning of the anterior–posterior axis, a phenotype highly reminiscent to the loss of Wnt3 in mice (Liu et al., 1999; Fu et al., 2009). The Wnt3 mutant phenotype is the earliest developmental abnormality among all Wnt knockouts, suggesting that the Gpr177-mediated Wnt production cannot be substituted. We have also demonstrated that *Gpr177*, activated by β-catenin and Lef/Tcf dependent transcription, is a direct target of Wnt (Fu et al., 2009). Upon Wnt activation, Gpr177 then assists the cellular trafficking of Wnt proteins in a positive feedback mechanism (Fu et al., 2009). This reciprocal regulation is required for establishment of the body axis during early embryogenesis. Our comprehensive survey of the Gpr177 mRNA and protein expressions has indicated that *Gpr177* may

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be involved in development of various organs (Yu et al., 2010). These results have led us to propose that reciprocal regulation of Wnt and Gpr177 is essential for Wnt-dependent development in health and disease.

To further determine the role of Gpr177 in controlling the developmental processes mediated by the Wnt pathway, we have created a new mouse strain permitting conditional inactivation of *Gpr177*. Genetic study further examines whether Wnt1-mediated development of the mouse embryo requires Gpr177 in addition to its essential role in Wnt3-mediated patterning of the embryonic axis. The ablation of Gpr177 in the Wnt1-expressing cells causes developmental deformities, much more severe than the Wnt1 knockout (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) but resembling the double knockout of Wnt1 and Wnt3a (Ikeya et al., 1997) and the conditional knockout of β-catenin (Brault et al., 2001). Although the ablation of Gpr177 does not recapitulate the Wnt1 knockout phenotype, our finding does support the theory for an overlapping function of Wnt family proteins present in the Wnt1-expressing cells. The Gpr177-dependent regulation of Wnt is likely to be critical for normal developmental and pathogenic processes of various organs.

RESULTS

Creation of the Gpr177Fx Allele Permitting Conditional Inactivation in Mice

To overcome the early embryonic lethality associated with the inactivation of *Gpr177* in mice (Fu et al., 2009), we created mice carrying a Gpr177Fx allele, permitting the ablation of Gpr177 by Cre-mediated recombination. We chose to insert loxP sites flanking exon 3 because its removal would cause an out-of-frame deletion, resulting in a null mutation. Four different mouse embryonic stem (ES) cell clones heterozygous for the targeted allele were obtained by homologous recombination (targeting efficiency: 4/48). Two of these targeted clones were used to generate mouse strains carrying the targeted allele. These strains were then crossed with the EIIa-Cre transgene to remove the pgk-neo cassette with or without the deletion of exon 3 to obtain mice carrying either Gpr177Δ or Gpr177Fx allele as illustrated (Fig. 1A). Polymerase chain reaction (PCR) analyses confirmed establishment of the Gpr177Fx and Gpr177Δ strains (Fig. 1B). Mice homozygous for Gpr177Fx allele were viable and fertile without any noticeable abnormalities, suggesting that insertion of the two loxP sites did not disrupt the *Gpr177* locus.

The Gpr177Fx Allele Is a Conditional Null Allele for *Gpr177*

Next, we examined whether the Gpr177Fx allele is a bona fide conditional null allele by examining the phenotypic defects associated with the germline deleted allele, Gpr177Δ. Similar to the $Gpr177^{lacZ}$ phenotype (Fu et al., 2009), we were not able to recover Gpr177Δ homozygous newborns or embryos after embryonic day (E) 10.5. The recovered Gpr177Δ homozygous embryos exhibited defects in formation of the anterior–posterior axis, identical to those observed in the $Gpr177^{lacZ}$ homozygote at E7.5 and E8.5 (Fig. 2). Whereas three germ layers developed in the controls (Fig. 2A,D,G), the Gpr177Δ (Fig. 2C,F,I) and $Gpr177^{lacZ}$ (Fig. 2B,E,H) embryos, lacking primitive streak and mesoderm, remained to grow as egg cylinders. Therefore, Gpr177Δ is a null allele, further indicating that the Gpr177Fx allele is a conditional null allele.

Deletion of Gpr177 by Wnt1-Cre Induces Brain and Craniofacial Abnormalities

We have previously shown that Gpr177 is essential for Wnt3-mediated establishment of the body axis (Fu et al., 2009). To test the generality of Gpr177 in the regulation of Wnt proteins, we carried out a genetic study to assess its role in the Wnt1-expressing cells. The Gpr177Fx allele was crossed with the Wnt1-Cre transgene to generate the Wnt1-Cre; Gpr177Fx/+ line. Intercross between the Wnt1-Cre; Gpr177Fx/+ mice and the Gpr177Fx/Fx mice obtained the Wnt1-Cre; Gpr177Fx/Fx ($Gpr177^{Wnt1}$) mutants. In these mutants, *Gpr177* was inactivated by the Wnt1-Cre transgene through Cre-mediated recombination. The $Gpr177^{Wnt1}$ mutants displayed brain abnormalities which are manifested at E10.5 (Fig. 3A–D). Craniofacial deformities were also obvious in the $Gpr177^{Wnt1}$ embryos at E13.5 (Fig. 3E,F) and E16.5 (Fig. 3G,H). Histology evaluation revealed the lack of mid/hindbrain structures in the mutants (Fig. 3I–T). In the craniofacial regions of $Gpr177^{Wnt1}$, several tissues derived from the cranial neural crest were impaired (Fig. 3M–T), suggesting that Gpr177 has a role in palatogenesis, tooth morphogenesis and development of the salivary and serous glands.

Gpr177 Is Required for Wnt-Mediated Brain Development

To further investigate the brain defects associated with conditional inactivation of Gpr177 by Wnt1-Cre, we first examined the expression of Wnt1 during embryonic brain development. At E9.5, Wnt1 is strongly expressed in the dorsal and ventral parts of the mesencephalon as well as the myelencephalon (Fig. 4A). The inactivation of Gpr177 in the Wnt1-expressing cells did not seem to affect the expression of Wnt1 in these regions (Fig. 4B). We were also able to detect similar levels of Wnt1, Wnt3/3a, and Wnt5a expression in the control and $Gpr177^{Wnt1}$ mutant embryos, suggesting that Gpr177 deficiency does not interfere with Wnt production (Fig. 4C). We then crossed the TOPGAL transgene, a reporter for β-catenin and Lef/Tcf dependent transcription, into the $Gpr177^{Wnt1}$ mutants. The TOPGAL transgenic activity was diminished in the developing brain of $Gpr177^{Wnt1}$, suggesting that Wnt/β-catenin signaling is affected by the Gpr177 deletion (Fig. 4D,E). These data are consistent with our previous finding that Wnt signaling, but not Wnt expression, is impaired by Gpr177 deficiency (Fu et al., 2009), thus suggesting a crucial role of Gpr177 in proper sorting and secretion of the Wnt proteins.

Next, we examined the expression of *En2*, which belongs to the engrailed family acting downstream of Wnt1 essential for mid/hindbrain development (Joyner et al., 1991; McMahon

et al., 1992; Wurst et al., 1994; Danielian and McMahon, 1996; Liu and Joyner, 2001). The En2-expressing domain almost disappeared in the Gpr177^{Wnt1} mid/hindbrain (Fig. 4F,G). In contrast, the Otx2 expression did not seem to be affected by the mutation in the forebrain (Fig. 4H,I), suggesting a region-specific effect of the Gpr177 deletion on brain development. This is also accompanied by dramatic reduction of the Fgf8 expression in the isthmic organizer of Gpr177^{Wnt1} (Fig. 4J,K). It has been shown that the Wnt-En signal is required for proper

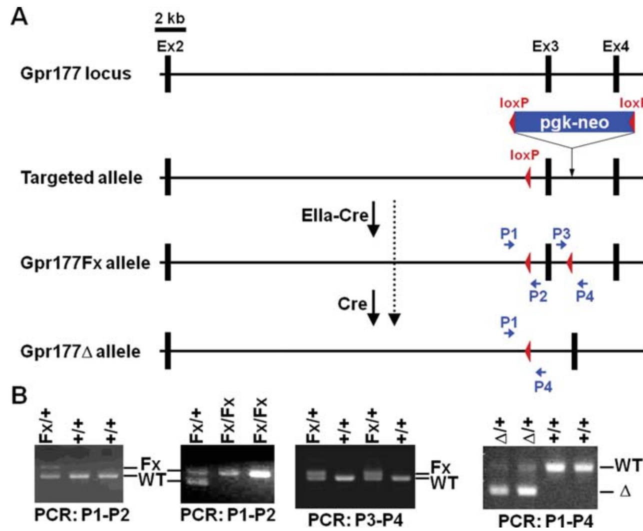

Fig. 1. Diagram illustrates our targeting strategy and the creation of mice carrying Gpr177Fx and Gpr177Δ allele. **A:** In the targeted allele, a loxP site and a pgk-neo cassette flanked by two loxP sites are inserted into intron 2 and intron 3, respectively. Mice carrying the Gpr177-targeted allele were crossed with the EIIa-Cre transgenic mice to generate progeny carrying the Gpr177Fx or Gpr177Δ allele. **B:** Polymerase chain reaction (PCR) analysis detects the presence of 5′ (PCR: P1–P2) and 3′ (PCR: P3–P4) loxP sites for genotyping the wild-type (+/+), heterozygous (Fx/+) and homozygous (Fx/Fx) mice, and examines the deletion of exon 3 in the Gpr177Δ/+ mice (PCR: P1–P4).

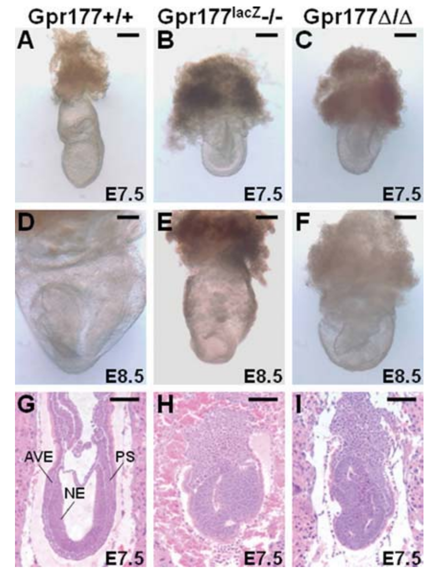

Fig. 2. The Gpr177Δ and Gpr177^{lacZ} homozygous embryos exhibit defects in establishment of the body axis. **A–I:** Gross morphological (A–F) and hematoxylin/eosin (H&E) staining (G–I) analyses of the embryonic day (E) 7.5 (A–C,G–I) and E8.5 (D–F) Gpr177+/+ (A,D,G), Gpr177^{lacZ}−/− (B,E,H), and Gpr177Δ/Δ (C,F,I) embryos reveal that the Gpr177Δ/Δ embryos show deficiencies in patterning of the anterior–posterior axis, identical to those observed in the Gpr177^{lacZ} homozygous embryos. AVE, anterior visceral endoderm; NE, neural ectoderm; PS, primitive streak. Scale bars = 200 μm in A–F; 100 μm in G–I.

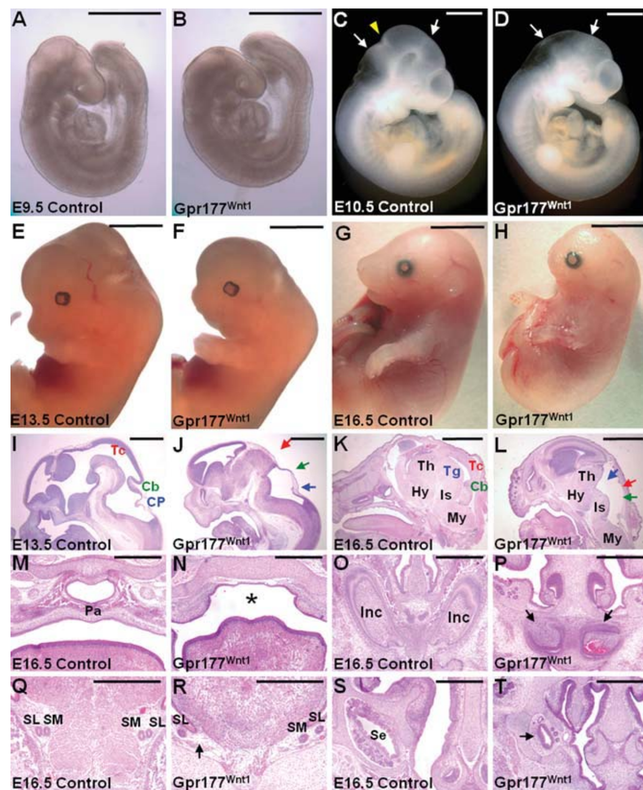

Fig. 3.

Fig. 3. The loss of Gpr177 in the Wnt1-expressing cells causes abnormalities in brain and craniofacial development. **A–T:** Gross morphological (A–H) and hematoxylin/eosin (H&E) staining (I–T) analyses of the embryonic day (E) 9.5 (A,B), E10.5 (C,D), E13.5 (E,F,I,J), and E16.5 (G,H,K–T) control (genotype: Wnt1-Cre; Gpr177Fx/+ or Gpr177Fx/Fx) and Gpr177^{Wnt1} (genotype: Wnt1-Cre; Gpr177Fx/Fx) littermates show developmental deformities in the mid/hindbrain and craniofacial structures caused by the deletion of Gpr177 in the Wnt1-expressing cells and their descendants. C,D: Arrows and arrowhead indicate the midbrain boundary and the truncated region, respectively. I–L: Arrows indicate the missing brain structures. M,N: Asterisk indicates cleft palate. O–T: Arrows indicate the tooth, salivary and serous gland defects. Cb, cerebellum; CP, choroid plexus; Hy, hypothalamus; Inc, incisor; Is, isthmus; My, myelencephalon; Pa, palate; Se, serous gland; SL, sublingual duct; SM, submandibular duct; Tc, tectum; Tg, tegmentum; Th, thalamus. Scale bars = 1 mm in A–D,I,J, 2 mm in E,F,K,L, 4 mm in G,H, 500 μm in M–T.

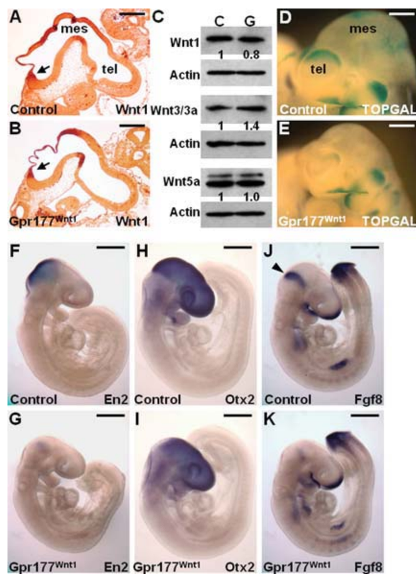

Fig. 4. Gpr177 is essential for Wnt-mediated neural development. **A,B:** Immunostaining of Wnt1 indicates its expression in the mesencephalon (mes) and myelencephalon (arrows) of embryonic day (E) 9.5 control (genotype: Gpr177Fx/Fx) and Gpr177^{Wnt1} (genotype: Wnt1-Cre; Gpr177Fx/Fx) embryos. **C:** Immunoblot analysis shows the expression levels of Wnt1, Wnt3/3a, and Wnt5a in the control (C) and Gpr177^{Wnt1} (G) mutant embryos at E9.5. The expression level of Actin was analyzed as a loading control. **D,E:** Whole-mount β-gal staining of E10.5 control (genotype: TOPGAL; Wnt1-Cre; Gpr177Fx/+) and Gpr177^{Wnt1} (genotype: TOPGAL; Wnt1-Cre; Gpr177Fx/Fx) embryos reveals that deletion of Gpr177 in the Wnt1-expressing cells impairs the expression of TOPGAL transgene in the telencephalon (tel) and mesencephalon (mes). **F–K:** Molecular marker analysis of control (genotype: Wnt1-Cre; Gpr177Fx/+ or Gpr177Fx/Fx) and Gpr177^{Wnt1} (genotype: Wnt1-Cre; Gpr177Fx/Fx) littermates at E9.5 characterizes the effects of the Gpr177 ablation on embryonic brain development using in situ hybridization of *En2* (F,G), *Otx2* (H,I), and *Fgf8* (J,K). Arrowhead indicates isthmic organizer. Scale bars = 200 μm in A,B, 500 μm in D–K.

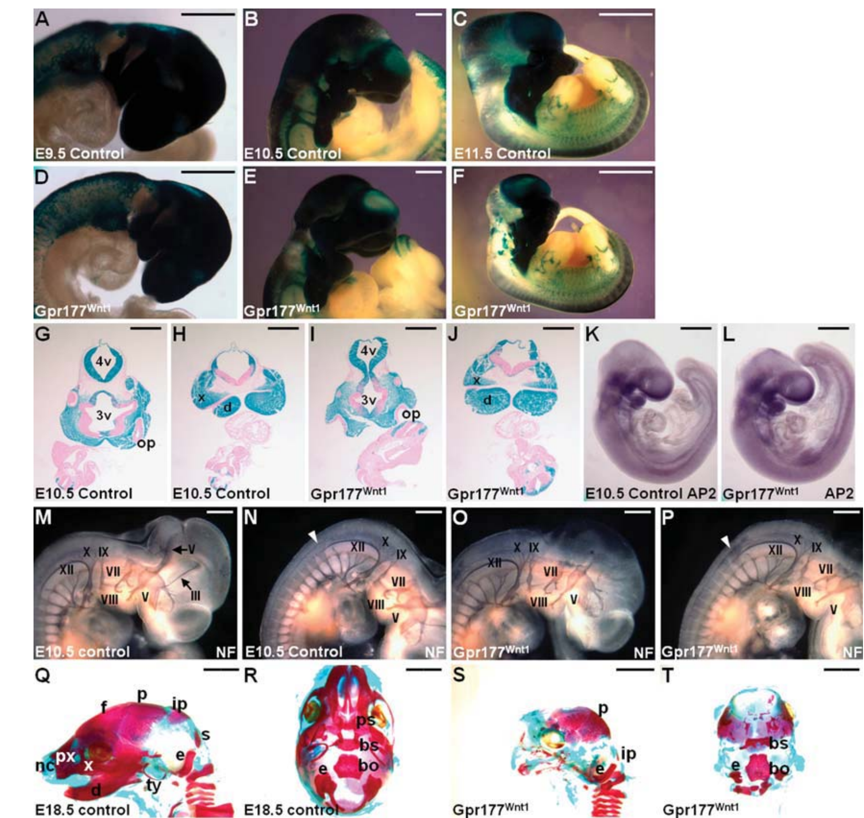

Fig. 5. Development of the neural crest derivatives are impaired by Wnt1-Cre–mediated inactivation of *Gpr177*. **A–J:** β-Gal staining of embryonic day (E) 9.5 (A,D), E10.5 (B,E,G–J), and E11.5 (C,F) embryos in whole-mounts (A–F) and sections (G–J) examines the neural crest cells which are descendants of the Wnt1-expressing cells in control (genotype: Wnt1-Cre; Gpr177Fx/+; R26RlacZ) and Gpr177^{Wnt1} (genotype: Wnt1-Cre; Gpr177Fx/Fx; R26RlacZ) littermates. **K,L:** In situ hybridization of AP2 labels cranial neural crest cells in the control and Gpr177^{Wnt1} embryos. **M–P:** Neurons of the control and Gpr177^{Wnt1} embryos were visualized by whole-mount immunostaining of neurofilaments in the cranial and trunk regions. Arrowheads indicate the dorsal root ganglions. Cranial nerves: III, oculomotor nerve; V, trigeminal ganglion; VII and VIII, combined ganglion of facial and vestibulocochlear nerves; IX, glossopharyngeal nerve; X, vagus nerve; XII, hypoglossal nerve. **Q–T:** Skeletal preparation of the E18.5 control and Gpr177^{Wnt1} littermates stained with Alizarin red and Alcian blue reveals severe abnormalities in the craniofacial skeleton caused by the Gpr177 deletion. Control genotype is Wnt1-Cre; Gpr177Fx/+ or Gpr177Fx/Fx and Gpr177^{Wnt1} genotype is Wnt1-Cre; Gpr177Fx/Fx. bo, basioccipital bone; bs, basisphenoid; d, mandible; e, exoccipital bone; f, frontal bone; ip, interparietal bone; nc, nasal cartilage; op, olfactory pit; p, parietal bone; ps, presphenoid; px, premaxillary bone; s, supraoccipital bone; ty, tympanic ring; x, maxillia; 3v, third ventricle; 4v, fourth ventricle. Scale bars = 500 μm in A–P, 2 mm in Q–T.

induction of Fgf8 in the isthmic organizer essential for patterning of the brain (Echevarria et al., 2005; Nakamura et al., 2005). Our results thus suggest that Gpr177 is essential for establishment of the isthmic organizer activity mediated by Wnt in mid/hindbrain development.

Craniofacial Deformities Caused by Inactivation of *Gpr177* in the Wnt1-Expressing Cells

Fate mapping analysis has previously suggested that the Wnt1-expressing cells behave as precursors for cranial neural crest cells during craniofacial morphogenesis (Chai et al., 2000; Jiang et al., 2000). Although the expression of Wnt1 is not detected in the postmigrated cranial neural crest cells, they are derivatives of cells expressing Wnt1 in the dorsal neural tube. To examine whether the loss of Gpr177 in these cells impairs cranial neural crest migration, we crossed the R26RlacZ allele (Soriano, 1999) into the Gpr177^{Wnt1} mice. Strong expression of lacZ was observed in the craniofacial regions of Gpr177^{Wnt1} from E9.5 to E11.5 (Fig. 5A–F). The lacZ reporter displayed a uniform expression pattern in the facial prominences (Fig. 5G–J). We did not observe any difference in the control and Gpr177^{Wnt1} embryos. Similar results were also obtained by the analysis of AP2, a neural crest marker (Fig. 5K,L). Our data suggest that the loss of Gpr177 does not cause migration defects. However, cranial nerves, which are derivatives of the neural crest, did not form properly in the Gpr177^{Wnt1} mutant (Fig. 5M–P). The skeletal development in both the viscerocranium and the neurocranium derived from the neural crest was also severely impaired

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(Fig. 5Q–T). The results thus suggest that Gpr177 plays an essential role in development of postmigratory neural crest cells during craniofacial morphogenesis.

DISCUSSION

Our previous study has shown that Gpr177 is required for Wnt3-mediated establishment of the body axis (Fu et al., 2009). By creating a conditional null allele, we are able to overcome the early lethality associated with the Gpr177 knockout, leading to an investigation of its role in other developmental processes involving the Wnt pathway. Using genetic analysis, we demonstrate that Gpr177 is essential for development of the mammalian head mediated by Wnt1. The loss of Gpr177 in the Wnt1-expressing cells not only impairs brain development, but also causes severe craniofacial deformities. These developmental defects are much more severe than the Wnt1 knockout (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), but highly reminiscent to phenotypes caused by the double knockout of Wnt1 and Wnt3a (Ikeya et al., 1997), and the β-catenin deletion in the Wnt1-expressing cells (Brault et al., 2001). Because the deletion of Gpr177 is likely to affect all Wnt sorting and secretion in the Wnt1-expressing cells, the $Gpr177^{Wnt1}$ phenotype thus reflects the impaired regulation of Wnt1 plus other Wnt(s). Our findings support the theory for the availability and the ability of other family members capable of compensating the loss of Wnt1. Gpr177-mediated regulation of Wnt is indispensable for craniofacial and brain development.

Although the loss of Gpr177 causes defects in craniofacial development mediated by neural crest cells, their induction and migration do not seem to be affected in the $Gpr177^{Wnt1}$ mutants. Fate mapping study has suggested that the Wnt1-expressing cells are precursors of the cranial neural crest (Chai et al., 2000; Jiang et al., 2000). However, Wnt1 is not expressed in the migrating and postmigratory neural crest cells, suggesting that Wnt signaling is repressed during the migratory process. This is consistent with our finding that Gpr177 is dispensable for neural crest cell migration. However, the development of postmigratory neural crest cells requires Gpr177, suggesting that Wnt signaling is essential for subsequent developmental processes during craniofacial morphogenesis.

In the course of preparing this study, Carpenter et al. reports the generation of a conditional null allele (Carpenter et al., 2010) similar to the one created by us. The difference between these two alleles is the targeting strategy where they insert two loxP sites flanking exon 1. It is not clear whether the loxP site insertion at the 5′ untranslated region interferes with the production of Gpr177. Indeed, the phenotypic defects associated with the Wnt1-Cre-mediated deletion described by Carpenter and colleagues seem more severe than those described in our study. In their mutants, the Gpr177 deletion induces a secondary defect in the telencephalon (Carpenter et al., 2010). Another possibility is that their analysis is performed in the heterozygous background where one copy of *Gpr177* has been inactivated in all cells (Carpenter et al., 2010). In our model, we inactivate Gpr177 in the Wnt1-expressing cells without manipulating its expression in cells which do not express Cre. If the differences between the two models are due to haploid deficiency, the gene dosage of *Gpr177* might be an important issue for the regulation of Wnt in development and disease.

Our comprehensive survey on the expression of Gpr177 has led to a hypothesis that reciprocal regulation of Wnt and Gpr177 is essential for the Wnt-dependent development of multiple organs (Yu et al., 2010). The Gpr177Fx mouse strain provides a powerful tool to further determine the requirement of Gpr177 in Wnt-mediated developmental and pathogenic processes. For the canonical pathway, there is now genetic evidence for the importance of Gpr177 in controlling Wnt1 and Wnt3 during mouse development. It is possible that Gpr177 is the master regulator in the signal-producing cells similar to the role of β-catenin, the master regulator in the signal-receiving cells, for the canonical Wnt pathway. Furthermore, the Wnt-producing cells are able to initiate autocrine and well as paracrine signaling effects, which add another layer of complexity to elucidate the regulatory mechanism. Whether noncanonical Wnt proteins are also regulated by Gpr177 remains an important issue to be addressed, especially by genetic analysis. Indeed, the brain and craniofacial defects exhibited in the $Gpr177^{Wnt1}$ mutants are somewhat similar to the Wnt5a null phenotypes (Yamaguchi et al., 1999). If Gpr177 modulates the sorting and secretion of canonical and noncanonical Wnt proteins, it becomes a real challenge to dissect the phenotypic defects associated with the Gpr177 deletion. This is because that both the canonical and noncanonical Wnt proteins could be expressed in the same cell. However, canonical and noncanonical signaling pathways may trigger different, and sometimes opposite, effects on tissue/organ development and maintenance. Studying the genetic interaction of Gpr177 and a specific Wnt signaling pathway promises new insights into the Gpr177-mediated regulation of Wnt in development and disease.

EXPERIMENTAL PROCEDURES

Mouse Strains

The Gpr177Fx ES cell lines were generated by electroporation of a targeting vector, containing the insertion of a loxP site in intron 2 and a pgk-neo cassette flanked by two loxP sites in intron 3, into CSL3 ES cells (Yu et al., 2005b; Chiu et al., 2008). Four mouse ES cell clones heterozygous for the targeted allele were obtained by homologous recombination (targeting efficiency: 4/48). Two independent clones were injected into blastocysts to generate chimeras that were bred to obtain mice carrying the targeted allele. These mice were then crossed with the EIIa-Cre transgenic mice to remove the pgk-neo cassette without or with the deletion of exon 3 to obtain the Gpr177Fx or Gpr177Δ mouse strain, respectively. Mice were genotyped by PCR analysis using primers (P1: 5′-TCCATTGAAGGCAAAACCTC-3′, P2: 5′-CTTTCATGGGCCATTTCAGT-3′) to identify the 5′ loxP locus, primers (P3: 5′-GCTGCTCTTG

AAGGACTTGTGTAGG-3′, P4: 5′-TGTTCATTGGTTCCTCTGGCTCTTA-3′) to identify the 3′ loxP locus and primers (P1: 5′-TCCATTGAAGGCAAAACCTC-3′, P4: 5′-TGTTCATTGGTTCCTCTGGCTCTTA-3′) to identify the exon 3 deleted locus. The Gpr177^{lacZ}, R26RlacZ, TOPGAL, and Wnt1-Cre mouse strains and genotyping methods were reported previously (Soriano, 1999; Yu et al., 2005a,b; Fu et al., 2009; Hsu et al., 2010). Care and use of experimental animals described in this work comply with guidelines and policies of the University Committee on Animal Resources at the University of Rochester.

Histology, β-gal Staining, Skeletal Analysis

Embryos were fixed, paraffin embedded, sectioned, and stained with hematoxylin/eosin for histological evaluation as described (Yu et al., 2007; Liu et al., 2008). Details for β-gal staining in whole-mounts and sections and for skeletal preparation and staining were described previously (Yu et al., 2005a,b, 2007; Maruyama et al., 2010).

In Situ Hybridization, Immunostaining, Immunoblot Analysis

In situ hybridization analysis was performed as described (Chiu et al., 2008; Fu et al., 2009). In brief, embryos were incubated with digoxigenin-labeled probes, followed by recognition with an alkaline phosphatase conjugated antidigoxigenin antibody (Roche). To visualize the bound signals, samples were incubated with BM-purple (Roche) for 4–5 hr. To generate RNA probes for in situ hybridization, DNA plasmids, containing AP2 (Mitchell et al., 1991), En2 (Joyner et al., 1991), Otx2 (Fu et al., 2009), and Fgf8 (Heikinheimo et al., 1994) cDNAs, were linearized for in vitro transcription using RNA polymerases T3 and T7 (Promega). For immunostaining analysis, the fixed embryos were incubated with primary antibodies which were detected with horseradish peroxidase-conjugated secondary antibodies followed by enzymatic color reaction according to the manufacturer's specification (Vector Laboratories) as described (Mark et al., 1993; Liu et al., 2007, 2008). Briefly, embryos were fixed in 4% paraformaldehyde for 6 hr at 4°C and treated with 1% hydrogen peroxide, 0.1% Triton X-100, 10% normal goat serum in phosphate buffered saline overnight at 4°C. Samples were then incubated with primary antibodies, followed by the addition of secondary antibodies conjugated with horseradish peroxidase. After extensive washing, samples were then stained for enzymatic color reaction. Cell extracts isolated form E9.5 embryos were subject to immunoblot analysis as described (Liu et al., 2008; Fu et al., 2009). Mouse monoclonal antibody neurofilament (2H3, Developmental Studies Hybridoma Bank), goat polyclonal antibodies Wnt1 (R&D Systems), Wnt3/3a (R&D Systems), and Wnt5a (R&D Systems) were used as primary antibodies as indicated.

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