Analysis of PRICKLE1 in human cleft palate and mouse development demonstrates rare and common variants involved in human malformations

Tian Yang1, Zhonglin Jia2,3,4, Whitney Bryant-Pike5, Anand Chandrasekhar5, Jeffrey C. Murray2, Bernd Fritzsch1 & Alexander G. Bassuk2

1Department of Biology, University of Iowa, Iowa City, Iowa, 52242
2Department of Pediatrics, University of Iowa, Iowa City, Iowa, 52242
3State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu, China
4Department of Cleft Lip and Palate Surgery, West China Hospital of Stomatology, Sichuan University, Chengdu, China
5Division of Biological Sciences, University of Missouri, Columbia, Missouri, 65211

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Correspondence
Alexander G. Bassuk, Department of Pediatrics, Division of Neurology, The University of Iowa, University of Iowa Children’s Hospital, 200 Hawkins Drive, 2500 JCF, Iowa City, IA 52242. Tel: 319-356-1851; Fax: 319-384-8818; E-mail: alexander-bassuk@uiowa.edu

Abstract
Palate development is shaped by multiple molecular signaling pathways, including the Wnt pathway. In mice and humans, mutations in both the canonical and non-canonical arms of the Wnt pathway manifest as cleft palate, one of the most common human birth defects. Like the palate, numerous studies also link different Wnt signaling perturbations to varying degrees of limb malformation; for example, shortened limbs form in mutations of Ror2, Vangl2looptail and, in particular, Wnt5a. We recently showed the noncanonical Wnt/planar cell polarity (PCP) signaling molecule Prickle1 (Prickle like 1) also stunts limb growth in mice. We now expanded these studies to the palate and show that Prickle1 is also required for palate development, like Wnt5a and Ror2. Unlike in the limb, the Vangl2looptail mutation only aggravates palate defects caused by other mutations. We screened Filipino cleft palate patients and found PRICKLE1 variants, both common and rare, at an elevated frequency. Our results reveal that in mice and humans PRICKLE1 directs palate morphogenesis; our results also uncouple Prickle1 function from Vangl2 function. Together, these findings suggest mouse and human palate development is guided by PCP-Prickle1 signaling that is probably not downstream of Vangl2.

Introduction
Cleft palate is one of the most common congenital birth defects. Cleft lip/palate (CL/P) was reported at a rate of 75.9 per 100,000 births in 2003, with 3066 cases reported in the US alone (Martin et al. 2005). Although strong data link maternal smoking to cleft palate in offspring (Shi et al. 2008), a wide range of congenital insults and genetic errors can lead to cleft abnormalities, leaving the etiology of cleft palate mostly unknown (Stanier and Moore 2004; Gritli-Linde 2007; Gritli-Linde 2008; Dixon et al. 2011).

Mice have been used as genetic models to study the etiology of cleft palate. In mice, around embryonic day 10.5 (E10.5), the secondary palate arises from the internal side of maxillary processes, first growing vertically along the side of the tongue (E12.5–E13.5) and then, around E14, growing upward and horizontally, populating the region above the tongue. At E15, the medial-edge epithelia of the two shelves fuse to render the continuous, intact palate (Gritli-Linde 2007; Bush and Jiang 2012). Disturbances during any of these stages can lead to cleft palate (Ferguson 1988).
Recently, several studies implicated Wnt signaling disruption in the pathogenesis of human palatal malformations (Chiquet et al. 2008; Menezes et al. 2010; He and Chen 2012; Mostowska et al. 2012a,b). For instance, single-nucleotide polymorphisms (SNPs) in WNT5A (wingless-type MMTV integration site family, member 5A) and ROR2 (receptor tyrosine kinase-like orphan receptor 2) have been linked to cleft palate in humans (Chiquet et al. 2008; Wang et al. 2012). Supporting the correlation in humans, both Wnt5a and Ror2 mutant mice have a complete cleft of the secondary palate (Schwabe et al. 2004; He et al. 2008). Clues to the underlying cause of cleft palate in Wnt5a and Ror2 mutant mice were revealed in the altered cell proliferation, and overall defective cell migration (He et al. 2008), while, cell death and in vitro palate fusion were normal in these lines (He et al. 2008). These data suggest that the cleft palate phenotype is the secondary effect of a problem with palatal growth.

Although it has been shown that Wnt5a/Ror2 phosphorylates Vangl2 (VANGL planar cell polarity protein 2) and thus regulates planar cell polarity (PCP) pathway in limb development (Gao et al. 2011), the role of Vangl2 in palate development seems to be limited. VANGL2 mutations have not been associated with human cleft palate. Consistent with this, Vangl2lox/C0 or Vangl2+/− mice do not have defects in palate closure (Kibar et al. 2001; Murdoch et al. 2001; Montcouquiol et al. 2003). In addition, Vangl2lox/C0 mutation does not increase the penetration of cleft palate in Fzd2+/− (frizzled family receptor 2, Wnt receptor) embryos, Fzd1+/− embryos, or Fzd1+/−; Fzd2+/− embryos (Yu et al. 2010). However, Vangl2lox/C0 mutation slightly increases the penetrance of cleft palate in Vangl2lox/C0; Fzd2+/−; Fzd7+/− mice (Yu et al. 2012). These results not only suggest the redundancy of Fzd and Vangl in palate development, but also raise the question as to whether all components of the PCP pathway are essential in palate development.

To answer this question, we started out to analyze the function of PRICKLE1 (prickle homolog 1, OMIM: 608500) in human and mouse palate development. The mammalian Prickle1 is believed to be a core PCP protein: it is thought to be recruited by Vangl to the cell membrane and this protein complex is asymmetrically localized at one side of the cell, which is the foundation to establish cell polarity (Gubb et al. 1999; Barrow 2006; Kestler and Kühl 2008; Raz et al. 2008; Tao et al. 2009; McNeill and Woodgett 2010; Gao et al. 2011; Wang et al. 2011; Yang et al. 2013). Our previous work in the limb development has shown that Prickle1, like Vangl2, Ror2 and Wnt5a, is essential for limb development (Yang et al. 2013). We also proposed that Prickle1 might mediate part of the Wnt5a, Ror2, Vangl2 signaling in limb development. Therefore, we examined whether Prickle1 is essential for palate development like Ror2, or it is only downstream of Vangl2, thus lacking a palate phenotype on its own.

The human population we analyzed was the Filipinos, who have high rates of CL/P. It was reported that CL/P occurs in 1.94 per 1000 live births, and 47,969 newborns with CL/P over an 8-year period at one hospital in Philippines in 1997 (Murray et al. 1997). This study also found high recurrence rates in siblings of nonsyndromic cleft lip/plate (NSCLP) in 23 per 1000 live births, and of cleft palate only (CPO) in 14 per 1000 live births (Murray et al. 1997). Furthermore, epidemiological, genome-wide association (GWA) studies and candidate genes studies on Philippine population identified several critical causal genes and environmental exposure factors for CL/P (Vieira et al. 2005; Beatty et al. 2010; Dixon et al. 2011; Ludwig et al. 2012). We show that mice homozygous for the Prickle1C251X mutation have cleft palate similar to Wnt5a or Ror2 mutants. This defect is associated with altered Shh expression. On the contrary, Vangl2 mutation does not affect Shh expression. In addition, we found linked common, noncoding variants in PRICKLE1 to cleft palate, and identified rare PRICKLE1 variants in patients with cleft palate. We conclude that, in contrast to limb development, during palate development the function of Vangl2 is uncoupled from Prickle1 function.

Material and Methods

Human data

Samples

All patient DNA samples were collected with written informed consent following internal review board criteria, abiding by the Helsinki Treaty, and de-identified. We sequenced the seven exons of PRICKLE1 in 87 nonsyndromic cleft lip and palate (NSCLP) individuals from Philippines. An additional 542 NSCLP individuals and 343 controls from Philippines were then similarly screened, to measure the frequency of the missense variants identified in the original screen (p.L380F [NM_153026.2:c.1138C>T] and p.R676W [NM_001144883.1:c.2026C>T]). Also, 221 large Filipino pedigrees (1032 nuclear families) with nonsyndromic cleft lip and palate (NSCLP) individuals from Philippines. Informed consent was obtained for all participants (University of Iowa approval numbers 199804081).

Sequencing

Primers were designed with Primer3 (http://biotools. umassmed.edu/bioapps/primer3_www.cgi) to cover all
Table 1. Primers of Prickle exons.

| Exons | Primer name | Sequence | Annealing temperature |
|-------|-------------|----------|-----------------------|
| EXON1 F | GGTCGGGGGTAAGAGAAATG | 60.0 |
| R | TGGTATTCCAGCATCTCACTG |
| EXON2 F | AGAGCCAAACTCCTGACCT | 60.0 |
| R | GGAGTTGGGGTTTATGAGCA |
| EXON3 F | TTCCCTTTTCTTAGAGGCTGTG | 60.0 |
| R | TGCTAGTCCAGTACCCCTACCC |
| EXON4 F | AGGAAAGCGCCAAATCGTGTG |
| R | ATTTTTGCTGATGTAACAGTGGGA |
| EXON5 F | TTAAGAGCCAGTTGCTGTC |
| R | CAAGCTCATCACGCTGAAC |
| EXON6 AF | GCTCCCCATACCCCAATAC | 60.0 |
| AR | TCGAGAAAGGTTGTCATA |
| BF | CAAGTTTCTGCTCCTCAG |
| BR | CAGTTCATCGTGACTGTCG |
| CF | CCAGAGCCTGCAATGAAAA |
| CR | ACTGCCTGCTGATAAT |
| EXON7 AF | TTGAGATTGGAAATTTCCTTTGAA | 54.7 |
| AR | TGCCGAGATTCATAGTCATA |
| BF | AAGGTAGGCTGGGAAGATGC |
| BR | TCAGAGAATACTGCCCTGTA |
| CF | TGATATGGTTTCTGCGGAC |
| CR | ACATGGGCAAGAAAGACCT |

Genotyping

Seven, tagging SNPs (rs12658, rs3747562, rs11181521, rs2406680, rs12309460, rs10880314, rs12581019) were genotyped using TaqMan SNP Genotyping Assays (Life Technologies, Grand Island, NY); and the results were analyzed with SDS 2.3 software (Applied Biosystems, Foster City, CA).

Statistical analysis

FBAT (v1.73) (Horvath et al. 2001) was used to perform the TDT analysis. Odds ratios for each SNP were calculated from PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/). To classify the types of variations, three criteria were set: very rare variants with an MAF less than 1%; rare variants with an MAF less than 5%; and common variants with an MAF above 5%. Significance levels adjusting for multiple comparisons using Bonferroni would be 0.05/28 (seven SNPs and four phenotypes).

Mice

All animal treatment was approved by University of Iowa IACUC (ACURF 0804066) and (ADURF1109204). The PrickleCys251X mutant mice have been previously described (Tao et al. 2011; Yang et al. 2013). Noon, on the day of the vaginal plug visualization, was designated as embryonic 0.5 (E0.5). Embryos were fixed in 4% paraformaldehyde (PFA). Mice were genotyped as previously described (Yang et al. 2013).

The Vangl2<sup>D255E</sup> mouse carrying the D255E mutation were originally obtained from Dr. Olivier Pourquié (IGBMC, Illkirch, France). Mice were phenotyped and genotyped as described previously (Glasco et al. 2012).

In situ hybridization

The probes for in situ hybridization were generated from the plasmid by in vitro transcription and then labeled with digoxigenin. Shh, Bmp4, Prickle1, Fgf10, and Wnt5a probes were previously described (Jones et al. 1991; Bittgood and McMahon 1995; Kraus et al. 2001; Pauley et al. 2003; Okuda et al. 2007; Glasco et al. 2012).

The embryos were hemi-sected in 0.4% PFA. For each probe, opposite halves from mutant and wild-type littermate embryos were labeled in the same tube, to minimize variability. The in situ hybridization protocol was previously described (Duncan et al. 2011). Whole-mount samples were digested with 20 μg/mL Proteinase K for 1 h. Each reaction was repeated at least once, at a given stage, until consistent results were achieved at least twice. Samples were imaged using a Leica M205 FA microscope with Leica Application Suite V3 (Wetzlar, Germany). All whole-mounted heads were imaged from the ventral side; and then images were compiled using CorelDRAW14.

Cryosection and H&E staining

Fixed mouse heads were incubated in 30% sucrose, 4% PFA, overnight, before sectioning. Samples were then frozen in OCT, in a tissue mold. Sections (20 μm) were cut in a cryostat at ~22°C, and then transferred to room temperature on microscope slides. H&E staining was then performed on the samples using the OMRF H&E staining protocol (http://imaging.omrf.org/wp-content/uploads/2012/09/HandE_Prototol.pdf). Samples were mounted in Permount and imaged using Nikon E800 microscope (Tokyo, Japan).
Proliferation test

Two hours before sacrifice, pregnant females were injected with PBS containing EdU (Invitrogen, Carlsbad, CA) at a concentration of 100 μg/gm body weight. Embryos were kept in 4% PFA, at least O/N, and sectioned with Microtome into 100 μm coronal sections. EdU Click-iT (Invitrogen) was performed on whole palate and corresponding coronal sections of wild-type and mutant palate, as per the instructions given by the manufacturer’s manual. Sections were imaged using a Leica SP5 confocal microscope. For coronal sections, three optical levels at a 5 μm distance were counted for each section; the counts were averaged and the average value was counted as the number for that sample. For whole-mount palates, 50 μm image stacks were taken at 5 μm interval from the ventral surface.

Quantification of palate length

First, a midline dividing the left and right head was defined. Objective markers were used to quantify the palate. Before the palate closure, the length of palate was measured from the junction between primary and secondary palate to the most posterior ruga which is labeled with Shh expression. After palate closure (E14.5), the length of the palate was measured from the posterior tip of the Shh expression in primary palate to the most posterior dot of Shh expression in the palatal shelves. A line parallel to this defined midline was drawn and the length of the line was measured in Corel Draw x 4. Left and right palatal shelves were measured separately and the average of the palate lengths was used as the palate length for that sample.

Table 2. The minor allele frequency of the variants.

| Position (Hg19) | rs ID | Amino acid change | Alleles | NSCLP-Philippine | Control-Philippine | 1000 Genome (CHB) | 1000 Genome (JPT) | Polyphen2/SIFT |
|----------------|------|-------------------|---------|------------------|-------------------|-------------------|-------------------|---------------|
| 42866332       |      |                   | T/C     | 0.59%            | –                  | –                 | –                 |               |
| 42863266       | rs79087668 | A124T          | C/T     | 1.79%            | 10.80%            | 5.60%             | –                 |               |
| 42863262       | rs34837068 | V125A          | A/G     | 11.90%           | 9.30%             | 5.60%             | –                 |               |
| 42859961       | rs12230583 |               | A/G     | 10.24%           | 23.70%            | 24.20%            | –                 |               |
| 42858525       |        | L380F*          | G/A     | 0.08%            | –                 | –                 | –                 | Benign/Deleterious |
| 42858698       |        | L380F*          | G/A     | 0.08%            | –                 | –                 | –                 | Benign/Tolerated |
| 42858137       | rs58707767 |                   | T/G     | 1.18%            | 4.10%             | 2.80%             | –                 |               |
| 42854208       | rs3747563 | F633F          | A/G     | 12.07%           | 30.40%            | 27.00%            | –                 |               |
| 42854205       | rs3747562 | S634S          | A/G     | 32.18%           | 45.40%            | 36.50%            | –                 |               |
| 42853871       | rs3827522 | P746S          | G/A     | 0.49%            | 10.30%            | 5.60%             | –                 | Benign/Tolerated |
| 42854081       | rs1043652 |               | C/T     | 0.61%            | –                 | –                 | –                 | Benign/Tolerated |

*The missense mutation was absent in 1000 Genome and NHLBI ESP.

Results

Rare PRICKLE1 variants in human cleft palate

To determine whether patients with nonsyndromic cleft lip and palate (NSCLP) could harbor variations in PRICKLE1, we first sequenced the entire coding region of PRICKLE1 in a cohort of 87 NSCLP patients from the Philippines. From a total of 629 patients and 343 ethnically matched controls, variants were identified and analyzed. This screen detected two families (A and B) each harboring a rare, PRICKLE1 variant that would be predicted to be deleterious—neither is represented in the 1000 Genomes Project, or the 8591 chromosomes from the National Heart Lung and Blood Institute (NHLBI) Exome Project; both were also absent in our Philippine controls (Table 2). Family A (Fig. 1A) harbors the p.L380F mutation (NM_153026.2:c.1138C>T), shared by the NSCLP-unaffected mother. PRICKLE1 c.1138C>T alters an evolutionarily conserved residue PRICKLE1p.L380F (Fig. 1A) that lies possibly within a phosphorylation site (predicted by http://www.cbs.dtu.dk/services/NetPhos/ and Xue et al. [2008]). Family B (Fig. 1B) harbors a p.R676W mutation (NM_001144883.1:c.2026C>T), also shared by the NSCLP-unaffected mother. PRICKLE1 c.2026C>T alters a highly evolutionarily conserved residue p.R676W (Fig. 1B), predicted to be within conserved nuclear localization signals (Shimojo and Hersh 2006) (http://www.sbc.su.se/~maccallr/nuc-pred/ and http://nls-mapper.iab.keio.ac.jp). Phosphorylation and nuclear localization signals are necessary for Prickle1’s localization to the nucleus, and thus these two
mutations possibly affected PRICKLE1’s normal function in the nucleus (Shimojo and Hersh 2003, 2006).

Although these two variants are rare, both were inherited from mothers without NSCLP, so they are not sufficient to cause NSCLP. If these variants contribute to NSCLP, then they do not show complete penetrance in either family. Such inheritance is consistent with previous reports of incomplete penetrance for NSCLP-associated genotypes, in both mice and humans (Juriloff 1982; Maestri et al. 1997; Parsons et al. 2008; Yu et al. 2010, 2012; Girardi et al. 2011; Nasser et al. 2012). The lack of any cleft palate abnormality in our previously described epilepsy patients with PRICKLE1 mutations further demonstrates that not all PRICKLE variations will be associated with palate abnormalities (Bassuk et al. 2008; Tao et al. 2011).

In addition to finding rare PRICKLE1 coding variants in the cleft palate cohorts, we evaluated the association between more common noncoding PRICKLE1 SNPs and cleft palate. Transmission disequilibrium test (TDT) results demonstrated that rs12658 (3′UTR of PRICKLE1) is associated with cleft lip only (CLO, P-value 0.004, marginally above the multiple comparisons P-value of 0.002.

Figure 1. Pedigrees of affected families, representative chromographs, and evolutionary conservation of altered PRICKLE1 amino acids. Red arrows denote affected nucleotides in respective chromographs. The highly conserved amino acids altered in both families are indicated by a red box. The family in (A) has (NM_153026.2:c.1138C>T p.L380F) mutation. The mother and son have the mutation. The family in (B) has (NM_001144883.1:c.2026C>T p.R676W) mutation. The mother and son have the mutation.
for significance), and the C allele at rs12658 is protective for CLO with OR = 0.61 (Tables 2 and 3). Taken together, these data suggest that both common noncoding variants and rare coding variants in PRICKLE1 may underlie palate malformations. To explore whether PRICKLE1 plays a role in causing NSCLP, we manipulated the Prickle1 gene in the mouse and then tracked palate development.

**Prickle1**<sup>C251X/C251X</sup> mice exhibit complete cleft secondary palate

To directly determine the role of Prickle1 in palate development, the palates of Prickle1<sup>C251X/C251X</sup> mutant mice (which harbor a stop codon mutation at cysteine 251 of the mouse Prickle1 protein) (Yang et al. 2013) were examined during the course of development. This mutation form of protein lacks the third LIM domain and the c-terminal phosphorylation sites and nucleus translocation signals, which are essential for nuclear localization of Prickle1 protein (Shimojo and Hersh 2003, 2006; Mapp et al. 2011; Yang et al. 2013). Therefore, the mutation in the Prickle1<sup>C251X/C251X</sup> mutant mice resembled the originally described mutation in human patients but, to an extreme extent.

At E18.5, all Prickle1<sup>C251X/C251X</sup> mutants had obvious shorter snout (Fig. 2A and B, compare black and red lines). We measured the length of the snout, which was defined from eye to tip of the snout, in a random selected subgroup of the embryos, and the mutants had ~10% shorter snout (<i>n</i> = 6, <i>t</i>-test, <i>P</i> < 0.05). When we examined the palate, the secondary palate was completely open in Prickle1<sup>C251X/C251X</sup> mice (Fig. 2C–F). In addition, the palate shelf of the mutant (arrow), often more deformed on the right palate shelf, was curved and farther from the midline (black dashed lines), compared to the left.

In Wnt5a mutants, the posterior palate shelves fail to rise to a horizontal position (He et al. 2008), so palate

Table 3. TDT results by cleft group.

| rs ID   | Position | Minor allele | CLO afreq | fam# | P** | NSCLP afreq | fam# | P** | NSCLP/P afreq | fam# | P** | ALL afreq | fam# | P** |
|---------|----------|--------------|-----------|------|-----|-------------|------|-----|---------------|------|-----|-----------|------|-----|
| rs12658* | 42853084  | C            | 0.45      | 71   | 0.0041 | 0.47  | 107  | 0.22 | 0.46  | 122  | 0.55 | 0.46  | 127  | 0.59 |
| rs3747562 | 42854205  | G            | 0.33      | 64   | 0.98  | 0.33  | 97   | 0.52 | 0.32  | 121  | 0.80 | 0.32  | 124  | 0.60 |
| rs11181521 | 42882367  | C            | 0.32      | 63   | 0.41  | 0.32  | 100  | 0.89 | 0.32  | 128  | 0.96 | 0.32  | 130  | 0.96 |
| rs24066680 | 42908378  | C            | 0.34      | 64   | 0.035 | 0.35  | 102  | 0.097| 0.34  | 122  | 0.86 | 0.34  | 123  | 0.96 |
| rs12309460 | 42933574  | A            | 0.29      | 60   | 0.55  | 0.30  | 97   | 0.85 | 0.30  | 111  | 0.90 | 0.30  | 117  | 0.76 |
| rs10880314 | 42904036  | C            | 0.35      | 68   | 0.28  | 0.35  | 110  | 0.058| 0.35  | 126  | 0.34 | 0.35  | 131  | 0.29 |
| rs12581019 | 42962542  | T            | 0.079     | 29   | 0.40  | 0.082 | 44   | 0.90 | 0.079 | 61   | 0.54 | 0.079 | 63   | 0.44 |

fam#, informative family number; # allele C is protective with OR = 0.61 from PLINK analysis; P**, P-value; ALL, CLO&NSCLP&CPO&Unknown cleft type.
elevation was examined in E18.5 embryos of Prickle1 mutant mice, in coronal sections of the palate. At this developmental stage, the wild-type palatal shelves had risen above the tongue and were fused at the midline (Fig. 3A). In the Prickle1C251X mutant, the palatal shelves had risen to horizontal but were too short to make the midline contact necessary for fusion (Fig. 3B). In some mutant mice, part of the palate shelves failed to rise normally (Fig. 3C).

Palatal mesenchyme expresses Prickle1 in an AP gradient

To begin to uncover the mechanism by which Prickle1C251X mutation caused cleft palate, the pattern of Prickle1 mRNA expression was visualized, by in situ mRNA hybridization of whole-mounted developing palates from E12.5 and E13.5 wild-type embryos. At E13.5, Prickle1 expression was detected in a gradient pattern that was higher in the posterior and lower in the anterior palate (Fig. 4A). To refine the topological view, the palate was sectioned coronally and tested with in situ hybridization. Here, Prickle1 expression was low in the anterior palate shelf, but was high in the mesenchyme of the posterior palate (Fig. 4A’–A”). The pattern of Prickle1 expression at E12.5 was similar to that at E13.5 (data not shown). As the mutant form of the mRNA is not stable (Frischmeyer and Dietz 1999; Chang et al. 2007; Yang et al. 2013), Prickle1 expression was expected to be lower in the mutants, as was the case in the developing limb (Yang et al. 2013). Consistent with this expectation, Prickle1 expression was weak in Prickle1C251X/C251X mice (Fig. 4B–B”), suggesting the reduced amount of Prickle1 protein in the mutant, if the protein is ever made.

The Prickle1C251X mutation affects Shh expression

Previously, we showed Prickle1C251X/C251X mutant limbs are perturbed for the expression pattern of Wnt5a and Bmp4, genes known to be important for palate development (Zhang et al. 2002; He et al. 2008; Yang et al. 2013). In addition, Wnt5a mutant mice expressed Bmp4 in an altered pattern but not Fgf10 (He et al. 2008). Accordingly, Prickle1C251X/C251X mice were assayed for the expression pattern of Wnt5a, Bmp4 and Fgf10, but neither whole-mount nor coronal section in situ mRNA hybridization in the E13.5 mutant palate detected obvious change from wild-type palate (Fig. 5). Thus, the Prickle1C251X mutation did not affect the expression pattern of the three genes.

Wnt5a, Bmp4 and Fgf10 are expressed in the mesenchyme but not epithelium; so we then examined the expression of Shh, which is well-known to mediate the epithelial-mesenchyme interaction during palate
development (Rice et al. 2004, 2006; Bush and Jiang 2012). Shh is expressed in the epithelium by the thickened rugae (Bitgood and McMahon 1995), which are transverse ridges on the secondary palate believed to aid in feeding and mastication (Bitgood and McMahon 1995). At E12.5, E13 and E13.5, Shh expression revealed the mutant palate developed fewer rugae compared to wild-type littermates (Fig. 6A–D, compare the number of arrows); however, by E15.5, both wild-type and mutant mice had developed the same number of rugae (Fig. 6E and F). Interestingly though, in E15 mutants, the medial edges of the anterior palatal shelves lacked Shh expression (Fig. 6G and H, asterisks), suggesting patterning abnormalities in the area. These results suggest that either rugae formation or Shh up-regulation was delayed in the mutant; however, this delay could be a consequence of the shorter palate shelves that had developed in the mutant.

The rugae provide objective markers to measure the length of the palate (Pantalacci et al. 2008; Welsh and O’Brien 2009), so we compared the length of palatal shelves in wild-type versus Prickle1C251X/C251X mice (see materials and methods for defining the boundary). From E12.5 to E15, the palatal shelves (Fig. 6A–H, compare red and black bars) of the mutant were about 80.3 ± 2.83% of the wild-type palatal shelves (n = 8, t-test, P < 0.01).
To understand whether the delay in Shh up-regulation in the mutant was a secondary effect from the shorter palate, the palate was examined at E11.5, a stage when the palate starts to develop (Gritli-Linde 2007). In the E11.5 mutants, the palates were shorter and curved differently than in wild-type littermates (Fig. 7A and B, dashed lines). Shh in situ hybridization offered insight into molecular changes at this stage: Shh was expressed weakly in the anterior palate; and, in the mutant, the Shh-positive region was already ~30% shorter than that of the wild-type palate (Fig. 7C and D, black and red bars). Shh was also only weakly expressed at the palate posterior tip (Fig. 7C and D). Unexpectedly, although the palates in Prickle1C251X/C251X embryos were already shorter, at this stage, we did not detect Prickle1 expression in the palate (Fig. 7E, dotted blue line). However, there was strong Prickle1 expression in the external maxillary processes that will develop into the cheeks and mouth (Fig. 7E). It is possible that Prickle1 is expressed weakly at this stage in the palate, below the detection threshold of whole-mount in situ hybridization. However, the shorter palate in the mutants at this stage suggests that Prickle1 has an earlier effect on palate development. Therefore, we examined Prickle1 expression 1 day earlier before the initiation of palate development at E10.5 (Fig. 7F). At this stage, Prickle1 was expressed by the internal maxillary processes (Fig. 7F, blue dotted line), which will extend medially into palatal shelves around E11.5. These results together suggest Prickle1 mutants have shorter palatal shelves due to shorter maxilla processes at the onset of the palate development.

**Proliferation rate is reduced in the posterior of Prickle1C251X/C251X palate**

The rugae are the organizational center of palate development. Cells in the rugae do not proliferate as fast as those cells between the rugae (Pantalacci et al. 2008; Welsh and O’Brien 2009). It is possible that this pattern in cell proliferation might be upset by delayed upregulation of Shh. To test this, cell proliferation was tracked with EdU in whole palate shelves. The images taken from the ventral side of E13.5 animals (Fig. 8A–A’ and D–D’) show that cells were more densely packed in the gap between the rugae (Fig. 8A and D, arrows); nevertheless, the densely packed cells comprising the rugae were mostly EdU-negative (Fig. 8A’ and D’, arrows). The wild-type and mutant mice showed no clear difference in the proliferation pattern. When proliferation was measured in coronal sections (Fig. 8B and C and E and F), fewer cells in the posterior palate were proliferating (Table 4). And as the posterior cells will migrate to the anterior (He et al. 2008), if fewer cells grow in the posterior palate, then ultimately, fewer will be available to migrate to populate the anterior palate. This could be the cause of the smaller palate in mutant mice.

In the developing limbs of Prickle1 mutants, we previously found that apoptosis changed more rapidly than in wild-type mutants (Yang et al. 2013). However, TUNEL test for apoptotic cells did not show any obvious changes in the apoptosis in the palate (data not shown).

**Vangl2lp mutation does not affect Shh expression**

Given that Vangl2lp mutants have shorter snouts similar to Prickle1C251X/C251X (Fig. 9A and B), we asked whether the palate AP growth was affected similarly and whether Shh expression was affected. Shh in situ hybridization was used to visualize the palate patterning (Fig. 9C–F). Interestingly, the mutant and wild-type palatal shelves were of...
almost equal length at E12.5, but by E14.5 the mutant shelves were shorter than those of the wild-type shelves. More importantly, the Shh expression pattern showed ru-gae formation was not affected at either E12.5 or E14.5 (Fig. 9C–F, white arrows). These results together suggest that a shorter snout alone does not cause cleft palate and that Vangl2 mutation does not affect Shh expression. This normal Shh expression in Vangl2looptail mutants also sup-ports that abnormal Shh expression might be the cause of smaller palate and thus cleft palate in Prickle1C251X mutants.

Discussion

**PRICKLE1 in human cleft palate**

Mutations in noncanonical Wnt signaling genes, such as WNT5A and ROR2, have been linked to human cleft palate (Chiquet et al. 2008; Wang et al. 2012) but this is the first study to examine human pedigrees for PRICKLE1 mutations associated with cleft palate. We identified two rare PRICKLE1 variants in individuals with NSCLP that are absent in controls. In addition, there is supportive evi-dence for an association of common variants in PRICKLE1 with NSCLP although this also does not reach genome-wide significance (Ludwig et al. 2012). This association in human cleft palate cases is supported by the strong phenoty-pne in Prickle1C251X/C251X mice.

**Possible interaction between Wnt5a/Ror2 and Prickle1**

The limbs of Wnt5a, Ror2, Vangl2 and Prickle1 mutant mice are shorter, to various extents, than those of wild-type counterparts (Yamaguchi et al. 1999; Raz et al. 2008; Gao et al. 2011; Wang et al. 2011; Yang et al. 2013), supporting the widespread idea that these four genes are part of the mammalian PCP pathway (Gubb et al. 1999; Barrow 2006; Kestler and Kühl 2008; Tao et al. 2009; McNeill and Woodgett 2010). Thus, we hypothesized that Prickle1-deficient mice would develop similar palatal defects as Wnt5a or Ror2 mutants. Consistent with our hypothesis (and Wnt5a as well as Ror2 data [Schwabe et al. 2004; He et al. 2008]), the Prickle1 mutation caused a completely cleaved palate (Fig. 1).
In mice, the posterior tip and anterior region of the palate express Wnt5a (Fig. 4), while Ror2 and Prickle1 are expressed in an opposite gradient along the palate (Fig. 3) (He et al. 2008). The overlapping expression of the three genes is consistent with the hypothesis that they are in the same signaling pathway. However, unlike the downregulation of Wnt5a expression in Prickle1C251X/C251X mutant limbs (Yang et al. 2013), Wnt5a expression did not appear to change in the mutant palate at E13.5. Therefore, if Wnt5a, Ror2 and Prickle1 interact in the palate, then they likely do so via a different mechanism than the one they use to interact in the limb.

Vangl2lp mutants do not develop cleft palate but had shorter snout. In addition, mice homozygous for mutant Vangl1 are viable and fertile; and Vangl1 knockout mice were not reported to have cleft palate (Torban et al. 2008). The clear involvement of Prickle1 but not of Vangl1/2 in palate development suggests that Prickle1 functions independently of Vangl1/2. Moreover, these results demonstrate that Prickle1 does not always interact with Vangl1/2 to mediate Wnt/PCP signaling.

Combined, our data suggest that, in the palate, Prickle1 might mediate the Wnt5a/Ror2 signal. How and why Vangl1/2 is uncoupled from this signal cascade in the palate remains unclear.

**Effect of Prickle1 on palate development**

At E10.5 before the development of palate, the internal maxillary processes express Prickle1. At E11.5, even though Prickle1 mRNA expression is not detected by whole mount in situ hybridization, the anterior palate of the mutant is already shorter than the wild-type palate. These results suggest Prickle1C251X mutation affects palate development partially by disrupted maxillary development. The shortened palate of mutant is further affected by delayed Shh upregulation or rugae formation, which is the signaling center of palate development (Welsh and O’Brien 2009).

Prickle1 is highly expressed by the posterior palate at E12.5 and E13.5. Supporting the role of Prickle1 in the posterior palate, we show proliferation is reduced in the posterior palate but not in the anterior palate. How does defective proliferation in the posterior palate lead to complete cleft of the whole secondary palate? One possible explanation is defective cell migration. It was shown that posterior palatal mesenchyme migrates anteriorly and anterior mesenchyme migrate medially (He et al. 2008). This migration requires Wnt5a/Ror2 signaling (He et al. 2008). If Prickle1 is part of this signaling, then the posterior mesenchyme probably cannot migrate anteriorly and the anterior mesenchyme cannot migrate medially. This leads to defects along AP axis and medial-lateral axis.

**Shh expression is affected in the Prickle1C251X mutation palate**

At E11.5, before the first ruga forms, we found that the Prickle1 mutant palate was already shorter than the wild-type palate. In addition, the Shh expression region is also affected by Prickle1C251X mutation.

Rugae are secondary signaling centers that coordinate the elongation of the palatal shelves (Welsh and O’Brien 2009). The rugae determine the expression pattern of several genes of the same signaling network (e.g., Notch1, Fgf9 and p63 [Welsh and O’Brien 2009]). In our Prickle1C251X mutation palate, we found that the Shh expression region is reduced in the posterior palate but not in the anterior palate. How does this affect Shh expression and what are the implications for palate development?
mutant palates, although Bmp4, Fgf10, and Wnt5a mRNA expression was unchanged, Shh expression was delayed, consistent with a slower overall growth of the mutant palate. As Shh upregulation and rugae formation require separation by a minimal distance (Pantalacci et al. 2008), the delayed rugae formation might be secondary to the short mutant palates. It is also possible that delayed rugae formation further delayed the formation of new signaling centers, which further impaired palate development. More data are needed to further reveal how these molecular interactions operate. Nevertheless, at later stages (after the wild-type palate has fused at the midline; E15), the mutant, unfused palate forms the same number of rugae as the wild-type palate, demonstrating that rugae development is not disrupted, only delayed. However, we noticed that in the anterior palate of the mutant, the medial edges of the palate shelves do not develop rugae, suggesting that the patterning is perturbed on medial edge of the palate.

**Differential growth effect of Prickle1 and Vangl2 on palate and snout AP growth**

Although both Prickle1 and Vangl2 affect palate and snout AP extension, they have different effects. Prickle1C251X mutants have shorter palatal shelves from E11.5 to E15, which suggests Prickle1C251X mutation has an early effect and a late effect on palate development: it causes smaller maxillary processes, which in turn leads to shorter palate; the shortened palate affects Shh expression, which further affects palate development. On the contrary, Vangl2lp mutation does not affect palate development until E12.5. After E12.5, Vangl2lp mutation starts to affect palate AP extension.

On the contrary, Vangl2lp mutation has similar effect on the AP extension of the snout to the Prickle1C251X mutation. The shortened snout but no palate closure defects in the palate of Vangl2lp mice compared with Prickle1C251X mice suggest shorter snout is not directly responsible for cleft palate.

In conclusion, we have shown that PRICKLE1 variants are associated with cleft palate in humans and a dysfunctional Prickle1 in mice causes a completely cleaved palate. This growth defect is associated with smaller maxillary processes, delayed rugae formation, and reduced proliferation in the posterior palate. However, Vangl2, classic partner of Prickle1 in the limb, is responsible for snout growth as Prickle1, but does not also cause cleft palate nor change the expression of Shh, which reveals that the function of Prickle1 can be uncoupled from Vangl1/2. Prickle1 adds to the recently discovered complexity of gene expression regulation in facial development (Attanasio et al. 2013) by showing a surprising flexibility in the use of what is usually considered consistent aspects of its signaling pathway.

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