Roles of Wnt pathway genes \textit{wls}, \textit{wnt9a}, \textit{wnt5b}, \textit{frzb} and \textit{gpc4} in regulating convergent-extension during zebrafish palate morphogenesis

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\textbf{ABSTRACT}

The Wnt signaling pathway is crucial for tissue morphogenesis, participating in cellular behavior changes, notably during the process of convergent-extension. Interactions between Wnt-secreting and receiving cells during convergent-extension remain elusive. We investigated the role and genetic interactions of Wnt ligands and their trafficking factors Wls, Gpc4 and Frzb in the context of palate morphogenesis in zebrafish. We describe that the chaperon Wls and its ligands Wnt9a and Wnt5b are expressed in the ectoderm, whereas juxtaposed chondrocytes express Frzb and Gpc4. Using \textit{wls}, \textit{gpc4}, \textit{frzb}, \textit{wnt9a} and \textit{wnt5b} mutants, we genetically dissected the Wnt signals operating between secreting ectoderm and receiving chondrocytes. Our analysis delineates that non-canonical Wnt signaling is required for cell intercalation, and that \textit{wnt5b} and \textit{wnt9a} are required for palate extension in the anteroposterior and transverse axes, respectively.

\textbf{KEY WORDS:} Wnt, Convergent-extension, Zebrabow, Palate, Morphogenesis

\textbf{INTRODUCTION}

Wnt signaling is a key pathway regulating tissue morphogenesis, notably through its role in the process of convergent-extension (CE), whereby cells medialize and intercalate in one axis with concurrent extension in the perpendicular direction (Gray et al., 2011; Heiseenberg and Tada, 2002; Heisenberg et al., 2000; Topczewski et al., 2011; Tudela et al., 2002; Wallingford et al., 2002; Westfall et al., 2003; Yin et al., 2009). The zebrafish ethmoid plate (hereafter palate) forms by the convergence and the integration of the midline frontonasal and paired maxillary prominences (Geetha-Loganathan et al., 2009; Kamel et al., 2013; Dougherty et al., 2013; Topczewski et al., 2001; Szabo-Rogers et al., 2010). The Wnt planar cell polarity (PCP) pathway has been implicated in both CE and craniofacial development but how it regulates cell behavior during palate morphogenesis remains elusive (Kibar et al., 2007; Sisson et al., 2015; Szabo-Rogers et al., 2010; Yang et al., 2014; Yin et al., 2009; Yu et al., 2010).

Wnts are highly conserved secreted proteins that share a common secretion factor, Wntlss (Wls), which is a multi-pass transmembrane protein that transports Wnt from the Golgi apparatus to the cell membrane (Bartscherer and Boutros, 2008; Brugmann et al., 2007; Franch-Marro et al., 2008; Garcia-Castro et al., 2002; Gleason et al., 2006; Harterink and Korswagen, 2012; Lee et al., 2008; Najdi et al., 2012; Wodarz and Nusse, 1998). Wnts show high hydrophobicity, which limits their free diffusion in the extracellular space. Extracellular matrix components, such as Glypican (Gpc4), ensure their diffusion to neighboring cells, while sFRP (Frzb) enhances diffusion of Wnt by blocking short-range effects and increasing long-range gene responses (Cadigan and Peifer, 2009; Gallet et al., 2008). At the receiving cell surface, Wnts bind to their receptor Frizzled (Fzd), activating an intracellular cascade of canonical β-catenin or non-canonical pathways (Chiquet et al., 2008; Clevers, 2006; He and Chen, 2012; Zecca et al., 1996).

We carried out morphogenetic analyses of the Wnt pathway from the epithelium (signaling cell) to the chondocyte (recipient cell) during palate development in zebrafish. We analyzed the requirement of genes encoding Wnt ligands (\textit{Wnt9a} and \textit{Wnt5b}), proteins involved in secretion (\textit{Wls}) and extracellular Wnt localization (\textit{Frzb} and \textit{Gpc4}). We detailed the relative contribution of each Wnt component to cellular behaviors mediating palate morphogenesis.

\textbf{RESULTS AND DISCUSSION}

\textbf{Requirement of Wnt pathway genes in palate morphogenesis}

We previously showed that \textit{wnt9a} is expressed in the oropharyngeal epithelium, whereas \textit{frzb} is expressed in the distal chondrocytes of the palate (Dougherty et al., 2013; Kanel et al., 2013). Consistent with the requirement for Wnt secretion, \textit{wls} is expressed in the epithelial and mesenchymal tissues surrounding the palate, colocalized with \textit{wnt9a} (Fig. S1A,F,K, black arrowheads; Fig. S1D,I,N, black and open arrowheads in E and O), and overlapping with \textit{wnt5b} in the epithelium lining the mouth opening (Fig. S1A,F,K, open arrowhead; Fig. S1E,J,O, black arrowheads in E and O). \textit{gpc4} is expressed more broadly, in both chondrocytes and epithelium (Fig. S1B, black arrowhead; Fig. S1G,L).

To elucidate the requirement of Wnt genes in palate morphogenesis, \textit{wls} (c186 allele) (Kuan et al., 2015), \textit{gpc4} (knypek) (Topczewski et al., 2001) and \textit{wnt5b} (pipetail) (Westfall et al., 2003) mutants were collected, and \textit{frzb} and \textit{wnt9a} mutants were generated (Hwang et al., 2013) (Fig. S2). We generated two alleles for each gene, confirming the specificity of their craniofacial phenotype. Analyses were conducted on \textit{wls\textsuperscript{c186}}, \textit{wnt9a\textsuperscript{c116,c118del}} and \textit{frzb\textsuperscript{c481,c487del}} alleles. Phenotypes were assessed by Alcian Blue staining of craniofacial skeletons and by measuring palate dimensions: length (L), width (W) and L/W ratio (Fig. 1).
For wild-type (WT) palate, the average L/W ratio was 1.15±0.08 (n=7; Fig. 1S). The wls mutant produced a shorter and wider palate with a L/W ratio of less than 1 (0.7±0.06, n=17; Fig. 1B,H,N,S). The morphology of mutant gpc4 cartilage was similar to wls−/−, although more severely dysmorphic (L/W ratio of 0.38±0.07, n=6; Fig. 1C,I,O,S). This was unexpected based on their function in Wnt trafficking, where Wls regulates all Wnt secretion and Gpc4 functions as a Wnt co-receptor for short-range diffusion. The greater severity of the gpc4 mutant phenotype compared with the wls mutant could be the consequence of earlier embryogenesis defects, since gpc4 mutants exhibited anomalies in body axis extension, or might be a consequence of its role in additional pathways (Filmus et al., 2008).

frzb mutants showed a discrete phenotype of slightly shortened palate (L/W ratio of 0.94±0.12, n=17; Fig. 1D,J,P,S), consistent with previous mouse work (Lories et al., 2007), but less severe than that observed with morpholino knockdown (Kamel et al., 2013; Dougherty et al., 2013). This difference between mutants and morphants has been reported previously as the field increasingly generates mutants, and might be explained by morpholino off-target effects or compensations that mitigate frzb mutation effects (Lodewyckx et al., 2012; Neumann et al., 2009; Panakova et al., 2005; Rossi et al., 2015).

Likewise, wnt9a−/− palate exhibited a modest phenotype compared with the morphant (Dougherty et al., 2013). The palate was elongated and narrower, but not significantly different from WT (L/W ratio of 1.19±0.15, n=21; Fig. 1Q,S). The wnt5b mutant showed a shorter palate than WT, but longer than wls mutant (Fig. 1F,L,R,S). Quantitatively, the L/W ratio was not affected (0.97±0.19, n=5). Consistent with their restricted expression patterns, the wnt9a and wnt5b mutant phenotypes suggest distinct requirements in palate morphogenesis. wnt5b is expressed in a discrete anterior domain and appears to be required for extension of the palate along the anteroposterior (AP) axis. wnt9a is expressed in the epithelium surrounding chondrocytes and is required in the extension of chondrocytes in the transverse axis of the palate.

Chondrocyte stacking reflects palate phenotype
Chondrocyte shape, orientation and stacking are crucial for craniofacial cartilage form and function (LeClair et al., 2009; Sisson et al., 2015; Sisson and Topczewski, 2009; Topczewski et al., 2001). Chondrocyte organization was assessed by measuring the L/W ratio (the longest cell axis measured as L, and the shorter axis as W) and the orientation as the angle between the AP axis of the palate and the longest axis of the cell. Finally, chondrocyte stacking was evaluated by the number of cell layers along the dorsoventral (DV) axis (Fig. 2Y).

In WT, chondrocytes organize as columns (Fig. 2G), oriented perpendicularly to the AP axis 90°-120°, as a single layer (Fig. 2M,S). Detailed view of mutant palates revealed significant differences in wls−/− and gpc4−/−. In the wls mutant, chondrocytes were smaller, rounded and lacked stacking in linear columns (Fig. 2B,H). Cells were randomly oriented (35°-145°, Fig. 2N) and exhibited excessive stacking in the DV axis (Fig. 2T). Similarly, gpc4 mutant chondrocytes showed defects in elongation and orientation and organized as a multi-layered structure (Fig. 2I,O,U). In frzb−/−, wnt9a−/− and wnt5b−/− embryos, cell orientation and stacking did not significantly differ from WT (Fig. 2P-R,V-X,Z).
**Fig. 2. Cell shape and orientation are defective in Wnt signaling mutants.** (A-F) Dissected palate (anterior to the top). (G-L) Representative region (as boxed above) magnified to illustrate cell shape and organization. (M-R) Cell orientation was measured and compared with WT (significantly different indicated by red asterisk) and with wls mutant (significantly different indicated by blue asterisk). Watson-U² test. (S-X) Transverse sections (following the cut plane illustrated in Y) showing chondrocytes stacking in the DV axis. (Z) Graphic representation of the cell L/W ratio. *P<0.05, ***P<0.0001.

**wnt9α−/−** chondrocytes exhibited a significantly increased L/W ratio (2.23±0.54 versus 1.95±0.49 for WT, Fig. 2Z) but stacked normally (Fig. 2W). *wnt5b−/−* exhibited smaller chondrocytes consistent with a Wnt-Ca²⁺ role in cell inflation (Hartmann and Tabin, 2000; Enomoto-Iwamoto et al., 2002). Moreover, *wnt5b* mutation affected the columnar organization but not the single layer stacking (Fig. 2L,X).

Detailed observation of *wls* mutant chondrocytes showed that Wnt signaling regulates palate morphogenesis through its effect on chondrocyte size, orientation and stacking. This analysis highlights the crucial role of Wnt-PCP, since *gpc4* mutation recapitulates most defects observed in *wls* mutants.

**wls mutation affects the CE mechanism**

Palate morphogenesis is mediated by the convergence and integration of facial prominences, followed by palate elongation through cell proliferation (Dougherty et al., 2013; Kamel et al., 2013). In other developmental contexts, Wnt-PCP signaling mediates the CE mechanism, driving axis elongation by cellular reorganization. To explore if a similar process is involved during palate morphogenesis under the control of Wnt, we observed cell behaviors in the *wls* mutant.

We first assessed cell proliferation using two independent methods: photoconversion cell labeling and EdU assays. As previously described, in the pulse-chase assay the photoconversion of Kaede protein, driven by the neural crest-specific *sox10* promoter, was performed. The entire distal edge of the palate was irreversibly photoconverted from green to red at 55 hpf, and the embryo was then allowed to develop and reimaged at 72 hpf. The palate is noted to have extended where the green chondrocytes are detected distally to the photoconverted region, indicating that new cells have been added along a distal front. This photoconversion assay illustrates extension of the lateral element of the palate via both proliferation and morphological changes (Fig. 3A,J) (Swartz et al., 2011; Dougherty et al., 2013; Kamel et al., 2013). In this assay, at 72 hpf WT and *wls* mutant showed comparable proliferation at the distal edge of the palate (Fig. 3B,K). Further, EdU labeling confirmed normal proliferation at the distal tip in both WT and *wls* mutant (Fig. 3C,L,Q). TUNEL assays and Acridine Orange staining did not reveal increased cell death in *wls* mutant compared with WT (not shown). Together, our data demonstrate that defects in cell proliferation and survival do not explain the shortened palate phenotype in the *wls* mutant.

Since cranial neural crest cells (NCCs) develop normally (Fig. S3) and chondrocytes survive and proliferate normally in the *wls* mutant, and the *wls* mutant palate exhibited increased cellularity in the DV axis (Fig. 2T), we examined how cell movements might be defective to give rise to the aberrant tissue architecture. We tested this hypothesis by visualizing cell rearrangements using the Zebrabow system (Pan et al., 2013; Vieira et al., 2005). The *Zebrabow* transgenic line enables labeling of clonal cell populations over time by allowing each cell and their descendants to express a unique combination of three fluorescent proteins: RFP, YFP and CFP (Livet et al., 2007; Pan et al., 2013; Rochard et al., 2015). WT *Zebrabow* palate depicted mediolateral intercalation along the AP axis (Fig. 3D-G,M-O) (Keller et al., 2008; Wallingford et al., 2002). Following the yellow clonal population, we were able to visualize CE occurring in three discrete steps (Fig. 3, from E, immature cells, to G, more mature cells): cells initially proliferate and aggregate distally at the newly formed part of the palate (Fig. 3E); cells then mature and organize into columns (Fig. 3F); and finally the chondrocytes intercalate proximally and drive elongation in the AP axis. (Fig. 3H-X).
axis, remaining as a single cell layer sheet in the DV axis (Fig. 3G). In contrast, these cell behaviors of intercalation and extension failed to occur in the wls mutant, as cells remained aggregated throughout the palate, chondrocytes did not intercalate with neighboring cells, and cells extended in the AP and DV axes (Fig. 3M-O).

It is commonly accepted that Wnt acts on neighboring cells (Strigini and Cohen, 2000). Since wls is only expressed in surrounding epithelial and mesenchymal cells (Fig. S1), it might regulate chondrocyte intercalation from these surrounding tissues. We assessed the ability of wls-deficient cranial NCCs to intercalate in a WT context using cell transplantations (Kemp et al., 2009). These experiments revealed that wls-deficient cells behave normally in a WT host (Fig. 3H compared with 3I), as they undergo normal intercalation and spread along the AP axis. Conversely, in a wls−/− host, WT cells failed to intercalate and spread along the AP axis, remaining grouped throughout the palate phenotype (Fig. 4D,J,S); the palate appeared narrower (average width 285 µm) and significantly improved cell orientation compared with mutants of downstream genes (gpc4, frzb, wnt5b and wnt9a) that the cell intercalation signal is provided by surrounding Wnt-expressing epithelial tissues in a non-cell-autonomous manner.

Together, these results suggest that Wnt signaling, provided by epithelium and mesenchyme tissues surrounding chondrocytes, is required for proper cell intercalation but not for cell proliferation during palate morphogenesis.

**Genetic interactions between wls, gpc4, frzb, wnt9a and wnt5b in the non-canonical Wnt pathway**

We next sought to examine genetic interactions between the Wnt genes described above and to validate the role of PCP by generating compound mutants (Fig. 4). We hypothesized that combining wls−/− with mutants of downstream genes (gpc4, frzb, wnt5b and wnt9a) would recapitulate the wls mutant phenotype.

An additive effect of mutations was not observed in the wls−/−; gpc4−/− compound mutant (Fig. 4C,I,O), suggesting that both genes act similarly on palate morphogenesis. The palate phenotype of the wnt9a−/−;wnt5b−/− double mutant appeared to be a combination of the respective single mutants, significantly different from wls−/− but not from WT (Fig. 4F,L,R). The addition of their respective phenotypes suggests that they might function independently. wnt9a−/−;wnt5b−/− did not recapitulate the wls−/− phenotype, implying that the Wnt-Ca2+ pathway (represented by wnt5b) and the Wnt9a signal are not involved in CE. Instead, this suggests the role of additional Wnt ligands, such as Wnt11 or Wnt4, in palate development (Lee et al., 2008; He and Chen, 2012).

The addition of frzb mutation to wls mutation partially rescued the palate phenotype (Fig. 4D,J,S); the palate appeared narrower (310 µm, as compared with 322 µm for wls−/− and 265 µm for WT; Fig. 4S) and the cell orientation significantly improved compared with wls−/−. One explanation of this partial rescue might be that compensation for the frzb mutation could improve Wnt ligand diffusion by lipoprotein addition (Neumann et al., 2009; Panakova et al., 2005). In this case, the compensation might also improve trafficking and short-range activity.

A partial rescue of wls mutation was also observed in the wls−/−; wnt9a−/− compound mutant; the palate appeared narrower (average width 285 µm) and significantly improved cell orientation compared with the wls mutant (Fig. 4E,K,Q,T). One explanation for this partial rescue is that there is Wls-independent Wnt trafficking, as previously reported in Drosophila (Ching et al., 2008). Another possibility is that...
wls and wnt9a, having similar expression patterns (Fig. S1), might regulate each other by regulatory feedback (Fu et al., 2009). Wnt9a might limit wls expression in chondrocytes; thus, by suppressing wnt9a, wls expression increases in chondrocytes, allowing local Wnt trafficking and subsequently some cell rearrangement.

Together, our results show that palate morphogenesis involves chondrocyte mediolateral interaction, subtly coordinated by wls, gpc4, wnt9a, wnt5b and frzb (Fig. 4U). Since CE facilitates the fusion of facial prominences and mediates palate extension, any Wnt dysregulation may be related to orofacial cleft pathogenesis.

MATERIALS AND METHODS
Animals
Mutant lines (gpc4^{hi1688Tg/+}, wnt5b^{hi2753tg/+}) were obtained from the Zebrafish International Resource Center (ZIRC). Wls^{186/186} mutants and the Zebrafish-M transgenic line were provided by Marnie Halpern, Carnegie Institution, Baltimore, MD, USA (Kuan et al., 2015) and Alex Schier, Harvard University, Cambridge, MA, USA (Pan et al., 2013), respectively. Animals were treated in accordance with IACUC guidelines. For further information, see the supplementary Materials and Methods.

Histological and morphological analysis
Cartilage was stained with Alcian Blue at 4 dpf (Walker and Kimmel, 2007). Length and surface area (μm) were measured with ImageJ (NIH) software. A minimum of three fish and 60 cells were measured.

Statistical analysis
All data are represented as mean±s.d. Significance was determined by one-way ANOVA and Tukey’s post-hoc test and attained at P<0.05 using Prism (GraphPad) or Oriana (Kovach) software. Watson-U^2 test was used for angular data and Student’s t-test or ANOVA for length/width measurements.

RNA in situ hybridization
Whole zebrafish embryos were stained by in situ hybridization as described (Thisse et al., 2004). Sections (10 μm) were prepared by OCT (Tissue-Tek, #4583) embedding.

Quantitative PCR (qPCR)
RNA isolated from whole zebrafish embryos was subject to qPCR analysis of frzb as described in the supplementary Materials and Methods.

Western blot
Detection of Wnt9a and Wls protein in WT and mutant embryos was performed as described in the supplementary Materials and Methods.

Proliferation and TUNEL assays
Proliferation and TUNEL assays were performed using the Click-IT assay (Thermo Fisher Scientific) following the manufacturer’s instructions. See the supplementary Materials and Methods.

Lineage analysis and time-lapse confocal imaging
Stained whole-mount zebrafish embryos were imaged using a Nikon AZ100 or 80i compound microscope. Anesthetized embryos were live imaged on a Nikon Ai scanning confocal microscope. For further details, see the supplementary Materials and Methods.

Generation of wnt9a, wls and frzb mutants
The wnt9a, wls and frzb mutants were generated by CRISPR-Cas9 techniques as described (Fu et al., 2013). For details, see the supplementary Materials and Methods.
Cell transplantation
Cells were transplanted as previously described (Kemp et al., 2009). Details are provided in the supplementary Materials and Methods.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
E.C.L. and L.R. conceived of the project and planned the experiments. L.R., S.D.M., I.T.C.L., R.H., M.H. and E.C.L. prepared the manuscript. All authors reviewed and revised the manuscript.

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