Published in final edited form as:

Dev Dyn. 2006 May ; 235(5): 1448–1454. doi:10.1002/dvdy.20723.

Expression of *Wnt9b* and activation of canonical Wnt signaling during midfacial morphogenesis in mice

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Abstract

Cleft lip with or without cleft palate (CLP) is the most common craniofacial birth defect in humans. Recently, mutations in the *WNT3* and *Wnt9b* genes, encoding two members of the Wnt family of signaling molecules, were found associated with CLP in human and mice, respectively. To investigate whether *Wnt3* and *Wnt9b* directly regulate facial development, we analyzed their developmental expression patterns and found that both *Wnt3* and *Wnt9b* are expressed in the facial ectoderm at critical stages of midfacial morphogenesis during mouse embryogenesis. Whereas *Wnt3* mRNA is mainly expressed in the maxillary and medial nasal ectoderm, *Wnt9b* mRNA is expressed in maxillary, medial nasal and lateral nasal ectoderm. During lip fusion, *Wnt9b*, but not *Wnt3*, is expressed in the epithelial seam between the fusing medial and lateral nasal processes. Furthermore, we found that expression of TOPGAL, a transgenic reporter of activation of canonical Wnt signaling pathway, is specifically activated in the distal regions of the medial nasal, lateral nasal and maxillary processes prior to lip fusion. During lip fusion, the epithelial seam between the medial and lateral nasal processes as well as the facial mesenchyme directly beneath the fusing epithelia strongly expresses TOPGAL. These data, together with the CLP lip phenotype in *WNT3*^{-/-} humans and *Wnt9b*^{-/-} mutant mice, indicate that *Wnt3* and *Wnt9b* signal through the canonical Wnt signaling pathway to regulate midfacial development and lip fusion.

Keywords

cleft lip; cleft palate; craniofacial development; facial morphogenesis; midface; lip fusion; TOPGAL; *Wnt3*; *Wnt9b*; Wnt signaling

Introduction

Cleft lip with or without cleft palate (CLP) has an occurrence of 1 in 500 to 2500 live births worldwide, which represents the most common craniofacial birth defect in humans (Vanderas, 1987; Schutte and Murray, 1999; Gorlin et al., 2001). The majority of CLP cases are nonsyndromic and the etiology is complex. Epidemiological studies consistently showed strong familial clustering and increased concordance in monozygotic twins (reviewed in Mitchell, 2002), indicating genetic predisposition to CLP. Genetic studies indicated that

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CLP is a multifactorial trait and recurrence patterns analysis suggested that there are likely three to fourteen CLP susceptibility loci in humans (Schliekelman and Slatkin 2002). The identity of these major CLP genes has remained elusive.

To facilitate identification of CLP susceptibility genes, several laboratories have carried out genetic analysis of CLP formation in mouse models because the morphogenetic and molecular processes of craniofacial development are strikingly similar in the mouse and human (e.g., Diehl and Erickson, 1997; Juriloff et al., 2001; Bush et al., 2004). One family of inbred mouse strains, the “A” strains, provides a particularly good model for understanding the genetic basis of nonsyndromic clefting because 5% to 30% of the newborns exhibit spontaneous CLP in different substrains and the genetic cause of this phenotype is determined by at least two interacting loci (Juriloff and Mah, 1995; Juriloff et al., 2001; 2004). A recessive gene, *clfl*, essential for CLP occurrence in the A strains was genetically mapped to distal chromosome 11 (Juriloff and Mah, 1995; Juriloff et al., 1996; 2001; 2004). Interestingly, the *clfl* region of mouse chromosome 11 is syntenic to human Chromosome 17q21, a chromosome region strongly associated with nonsyndromic CLP in some human populations (Chenevix-Trench et al., 1992; Shaw et al. 1993; Mitchell 1994; Peanchitlertkajorn et al., 2003; Marazita et al. 2004; Moreno et al., 2004). The *clfl* region contains two closely linked genes, *Wnt3* and *Wnt9b*, encoding members of the Wnt family of secreted cysteine-rich glycoproteins (Juriloff et al., 2004; 2005). Interestingly, Niemann et al. (2004) recently showed the correlation of a nonsense mutation (Q83X) in the *WNT3* gene with tetra-amelia and CLP in a large consanguineous family, indicating that *Wnt3* plays important roles in craniofacial development.

The Wnt family contains 19 members in humans and mice. Wnts can bind cell surface receptors of the Frizzled (Fzd) family and signal through several different intracellular signal transduction pathways to regulate diverse developmental processes, including cell proliferation, cell polarity, cell fate determination, and patterning of the neural tube and limbs (reviewed in Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). The best characterized Wnt signaling pathway, termed the canonical Wnt pathway, involves stabilization and nuclear transportation of β -catenin. In cells that do not receive a Wnt signal, cytoplasmic β -catenin is rapidly degraded through the ubiquitin-proteasome pathway. In cells responding to canonical Wnt signaling, β -catenin is stabilized and enters the nucleus to activate the Tcf/Lef family transcription factors and regulate transcription of downstream genes. Although several *Wnt* and *Fzd* genes as well as *Tcf1* and *Lef1* have been shown to be expressed in the developing facial primordia (Gavin et al., 1990; Oosterwegel et al. 1993; Parr et al., 1993; Christiansen et al., 1995; Wang and Shackleford, 1996; Borello et al., 1999), a role for Wnt signaling in facial morphogenesis was not known until the identification of the *WNT3* mutation in humans (Niemann et al., 2004) and a recent report of the *Wnt9b*^{-/-} mutant mouse phenotype that included incomplete penetrance of CLP (Carroll et al., 2005). Whereas no coding mutation in either the *Wnt3* or *Wnt9b* genes has been found in the A strains, a retrotransposon insertion has been identified approximately 6.6 kb 3' to the *Wnt9b* gene specifically in the *clfl* allele (Juriloff et al., 2005). This retrotransposon is present in all the cleft mouse strains containing the *clfl* allele and is not present in *CBA/J*, the ancestral strain to the A strains, indicating that the *clfl* mutation most likely causes CLP by interfering with *Wnt9b* gene expression (Juriloff et al., 2005). However, a report of the spatiotemporal expression patterns of either *Wnt3* or *Wnt9b* during craniofacial development is lacking and it is not known whether these genes directly regulate facial morphogenesis or whether the CLP phenotype results from secondary effects of the mutations. Moreover, although *Tcf1* and *Lef1* are expressed in the developing facial primordia (Oosterwegel et al. 1993), it is not known whether the canonical Wnt signaling pathway is actively involved in facial morphogenesis. We report here that both *Wnt3* and *Wnt9b* are expressed in the

developing facial ectoderm and that the canonical Wnt signaling pathway is activated during facial outgrowth and lip fusion.

Results and Discussion

Expression of *Wnt3* and *Wnt9b* during craniofacial development

Since the *clfl* mutation mostly likely affects the *Wnt9b* gene and since targeted disruption of *Wnt9b* causes CLP in mice (Carroll et al., 2005; Juriloff et al., 2005), we first examined *Wnt9b* mRNA expression during craniofacial development by using whole mount in situ hybridization of mouse embryos. By whole mount in situ hybridization analysis, *Wnt9b* mRNA expression is detected in the surface ectoderm covering the head and the branchial arches at E9.5 (Fig. 1A). Whereas the whole mount hybridization signal appears diffuse at this stage, specific *Wnt9b* mRNA expression in the surface ectoderm of the head and branchial arches is confirmed by in situ hybridization of paraffin sections of E9.5 embryos (Fig. 2A). By E10.5, *Wnt9b* mRNA is highly expressed in the facial primordia (Fig. 1B), in addition to strong expression in the Wolffian duct and the genital tubercle of the urogenital system as previously reported (Carroll et al., 2005). Facial views of the embryonic head region at this stage shows that *Wnt9b* mRNA is most strongly expressed in the distal regions of the medial and lateral nasal processes and of the adjacent maxillary processes (Fig. 1C). *Wnt9b* mRNA is also strongly expressed in the surface ectoderm of the mandibular processes (Fig. 1C).

Since CLP is part of the phenotype in *WNT3*^{-/-} human fetuses with tetra-amelia and since the *Wnt3* gene is closely linked to and shares the upstream region with the *Wnt9b* gene in both mice and humans, we also investigated the expression pattern of the *Wnt3* gene. At E9.5, *Wnt3* mRNA expression is detected in the tailbud but not in the developing head region (Fig. 1D). At E10.5, *Wnt3* mRNA is expressed in the surface ectoderm of the distal medial nasal processes and the maxillary and mandibular processes, partially overlapping with *Wnt9b* mRNA expression in the facial primordia (Fig. 1E, F). In contrast to *Wnt9b* expression, *Wnt3* mRNA is absent from the ectodermal contact sites between the medial and lateral nasal processes (arrowheads in Fig. 1C, F). This is further demonstrated by in situ hybridization of frozen sections of E10.5 and E11.5 mouse embryos. At E10.5, *Wnt9b* mRNA is expressed in the pre-fusion medial and lateral nasal ectoderm (Fig. 1G). This ectodermal expression of *Wnt9b* mRNA persists during fusion between the medial and lateral nasal processes at E11.0 (Fig. 1H). Together with the craniofacial defects observed in *WNT3*^{-/-} human and *Wnt9b*^{-/-} mice (Niemann et al., 2004; Carroll et al., 2005), these data indicate that *Wnt3* and *Wnt9b* play distinct roles in midfacial development.

To gain further insight into the role of *Wnt9b* in craniofacial morphogenesis, we carried out in situ hybridization of paraffin sections of mouse embryos at different stages using radioactively labeled cRNA probes. Consistent with the whole mount in situ hybridization data shown in Fig. 1, *Wnt9b* mRNA is detected in the surface ectoderm of the head and the branchial arches at E9.5 (Fig. 2A). By E10.5, *Wnt9b* mRNA expression is significantly increased in the ectoderm of the facial primordia, including the maxillary and mandibular processes (Fig. 2B, C). Expression of *Wnt9b* mRNA is also detected in the maxillary mesenchyme at this stage (Fig. 2C). By E11.5, *Wnt9b* mRNA is strongly expressed in a highly restricted pattern in the facial ectoderm of the nasal, maxillary, and mandibular processes, including the epithelial seam between the fusing medial and lateral nasal processes (Fig. 2, D–F). *Wnt9b* mRNA expression is not detectable from the facial mesenchyme by E11.5 (Fig. 2E, F). By E12.5, *Wnt9b* mRNA expression in the facial ectoderm is substantially down-regulated but remained detectable above background levels throughout late embryogenesis (Fig. 2G, H). The spatiotemporal pattern of *Wnt9b* mRNA expression, together with the CLP phenotype in the *Wnt9b*^{-/-} mutant mice, indicate that

Wnt9b plays critical roles in midfacial morphogenesis through regulating outgrowth of the facial processes and/or fusion between the medial and lateral nasal processes. *Wnt9b* mRNA expression is not detected in the secondary palate throughout its development (Fig. 2G, and data not shown), suggesting that the cleft palate phenotype observed in the *Wnt9b*^{-/-} mutant mice is a secondary effect of disruption of earlier facial developmental processes in these mutants.

The canonical Wnt signaling pathway is activated during lip fusion

Previous reports suggested that both Wnt3 and Wnt9b act through the β -catenin-mediated canonical signaling pathway during limb and urogenital development, respectively (Barrow et al., 2003; Carroll et al., 2005). To investigate whether the canonical Wnt signaling pathway is activated during facial primordial outgrowth and lip fusion, we examined the developmental expression patterns of *TOPGAL*, a transgenic *lacZ* reporter gene specifically activated in response to Tcf/Lef-mediated transcriptional activation by nuclear β -catenin (DasGupta and Fuchs, 1999; Merrill et al., 2004). At E9.5, expression of β -galactosidase activity from the *TOPGAL* transgene was detected in the midbrain and the migrating neural crest cells, including the trigeminal and facioacoustic ganglia and the neural crest streams migrating toward the branchial arches (Fig. 3A). Patchy expression of β -galactosidase activity was also detected in the forelimb ectoderm at this stage (Fig. 3A). By E10.5, strong β -galactosidase activity was detected in the dorsal telencephalon and mesencephalon, the cranial ganglia, and the apical ectodermal ridge of the limb buds (Fig. 3B). Strong β -galactosidase activity was also detected in the distal regions of the nasal, maxillary and mandibular processes at this stage (Fig. 3B, D). X-gal staining of cryostat sections of E10.5 embryos showed that *TOPGAL* is expressed in the ectoderm of the medial and lateral nasal processes as well as in the facial mesenchyme immediately adjacent to the *lacZ*-positive ectoderm (Fig. 3G). Between E11.0 and E11.5, the medial and lateral nasal processes fuse to each other in a proximal to distal zipper sequence (Trasler, 1968). Strong *lacZ* staining was detected in the pre-fusion medial and lateral nasal ectoderm as well as in the epithelial seam between the fusing nasal processes (Fig. 3H–J). Strong *lacZ* expression was also detected in the nasal mesenchyme adjacent to the expressing ectoderm in E11.5 and E12.5 embryos (Fig. 3E, F, I, J). However, no expression of the *TOPGAL* reporter transgene was detected in the developing palatal shelves of the secondary palate at these stages (data not shown). Thus, the expression pattern of the *TOPGAL* transgene in the developing facial primordia correlates with the *Wnt9b* mRNA expression pattern and indicates that the canonical Wnt signaling pathway is activated in both the facial ectoderm and adjacent facial mesenchyme during midfacial morphogenesis.

Development of the midface and formation of the intact upper lip depend on reciprocal signaling interactions between the ectoderm and the neural crest-derived mesenchyme in the facial primordia (Wedden, 1987; Richman and Tickle, 1989; Ashique et al., 2002; reviewed in Francis-West et al., 1998; 2003). Several major signaling pathways, including Bmp, Fgf, and Shh pathways have been shown to play essential roles in midfacial outgrowth and upper lip morphogenesis (Hu and Helms, 1999; Trumpp et al., 1999; Ashique et al., 2002; Trokovic et al., 2003; Jeong et al., 2004; Liu et al., 2005). In particular, tissue specific inactivation of either *Bmp4* or *Bmpr1a* results in failure of lip fusion in mice (Liu et al., 2005). Our data showing expression of both *Wnt9b* mRNA and *TOPGAL* activity in the pre-fusion and fusing lip ectoderm, together with the CLP phenotype in *Wnt9b*^{-/-} mutant mice (Carroll et al., 2005), suggest that Wnt9b activates the canonical Wnt signaling pathway to regulate lip fusion. Interestingly, both *Wnt9b* mRNA and *TOPGAL* expression overlap with the previously reported domains of *Bmp4* expression in the distal ectoderm of the facial primordia prior to and during lip fusion (Gong and Guo, 2003; Liu et al., 2005). In several developmental processes, including during development of the lung and limbs, Wnt/ β -

catenin signaling has been shown to act upstream of *Bmp4* (Barrow et al., 2003; Soshnikova et al., 2003; Shu et al., 2005). Moreover, the *Bmp4* gene promoter contains several Tcf/Lef binding sites and cell transfection studies have shown direct activation of the *Bmp4* promoter by activated canonical Wnt signaling (Shu et al., 2005). Thus, it is tempting to suggest that Wnt9b may act upstream of Bmp4 signaling to regulate lip fusion.

Methods

In situ hybridization and X-gal staining

Mouse embryos were collected at E9.5, E10.5 and E11.5, fixed in 4% paraformaldehyde overnight at 4 °C. Whole mount in situ hybridization was carried out as previously described (Jiang et al., 1998). For non-radioactive section in situ hybridization, embryos were washed in cold PBS, embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnology Inc.), and cryosectioned at 10 µm thickness. Sections were refixed with 4% paraformaldehyde for 20 minutes, digested with proteinase K, refixed, washed and hybridized with digoxigenin-labeled *Wnt3* or *Wnt9b* cRNA probes. Unbound probes were removed with stringent washes and the hybridization signals detected as previously described (Li and Joyner, 2001). The slides were further counterstained with nuclear fast red to visualize morphology. Section in situ hybridization using radioactive cRNA probes was carried out as previously described (Lan et al., 2001).

Expression of the *TOPGAL* transgene was detected by using X-gal staining. Timed matings of wildtype ICR female mice and *TOPGAL* transgenic male mice (purchased from the Jackson Laboratory, Bar Harbor, Maine) were set up and embryos dissected at E8.5 to E13.5. Fixation and X-gal staining procedures were performed as described previously (Jiang et al., 1998; Lan et al., 2004).

Acknowledgments

Grant Sponsor: NIH; Grant number: DE015207; Grant number: DE016215; grant number DE015291.

RCR is co-first author for this paper. We thank Tom Carroll and Andy McMahon for the *Wnt3* and *Wnt9b* cDNA probes. We thank Hiroyuki Mishima and Brandon Newell for assistance. This work was supported by NIH/NIDCR grant DE015207 to RJ and by NIH/NIDCR grants P50DE016215 and KO2DE015291 along with a University of Iowa College of Dentistry seed grant to ACL.

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Fig. 1.

Expression of *Wnt9b* and *Wnt3* mRNAs during craniofacial development in mice. (A) Lateral view of an E9.5 embryo showing *Wnt9b* mRNA expression in the head ectoderm. (B) Lateral view of an E10.5 embryo showing *Wnt9b* mRNA expression in the nephric duct (arrow), the genital tubercle (arrowhead), and in the facial ectoderm. (C) Facial view of the embryo in B showing strong *Wnt9b* mRNA expression in the distal epithelium of the medial nasal, lateral nasal, and maxillary processes as well as in the rostral surface of the mandibular processes. Arrowhead points to site of future lip fusion. (D) Lateral view of an E9.5 embryo hybridized with the *Wnt3* cRNA probe. *Wnt3* mRNA is expressed in the tailbud but not in the craniofacial region at this stage. (E) Lateral view of an E10.5 embryo hybridized with the *Wnt3* cRNA probe. *Wnt3* mRNA expression at this stage is weak except in the distal facial ectoderm. (F) Facial view of the embryo in E showing relatively abundant *Wnt3* mRNA expression in the maxillary and rostral mandibular processes. Weak expression is seen on the oral surface of the medial nasal processes but not at the site of contact between the medial and lateral nasal processes (arrowhead). (G) Frontal section through the pre-fusion medial and lateral nasal processes of an E10.5 embryo shows that *Wnt9b* mRNA expression (blue staining) is restricted to the distal epithelia (arrowheads) in both processes. (H) At E11.0, when the medial and lateral nasal processes begin to fuse with each other, *Wnt9b* mRNA is expressed in the epithelial seam (arrowhead). ba2, second branchial arch; fl, forelimb bud; fnp, frontonasal process; hl, hindlimb bud; Inp, lateral nasal process; man, mandibular process; max, maxillary process; mnp, medial nasal process.

Fig. 2.

Section in situ hybridization analysis of *Wnt9b* mRNA expression during craniofacial development. mRNA signal is shown in red and cell nuclei are counterstained blue. (A) Sagittal section of an E9.5 embryo showing *Wnt9b* mRNA expression in the head ectoderm (arrow) as well as in the surface and neural ectoderm in the tail region. Weak expression is also detected in the ectoderm of the mandibular processes (arrowhead). (B) Sagittal section of an E10.5 embryo showing strong *Wnt9b* mRNA expression in the ectoderm covering the maxillary and mandibular processes. (C) Frontal section of an E10.5 embryo showing *Wnt9b* mRNA expression in the maxillary ectoderm and mesenchyme as well as in the mandibular ectoderm. (D) At E11.5, *Wnt9b* mRNA is strongly expressed and highly restricted in the facial ectoderm, including the epithelial seam between the fusing medial and lateral processes (arrowhead). Arrow points to moderate levels of *Wnt9b* mRNA in the ectoderm covering the genital tubercle. (E) Higher magnification view of the head region of the embryo section shown in D. Arrowhead points to the epithelial seam between the fusing medial and lateral nasal processes. (F) Frontal section through the distal region of the facial primordia of an E11.5 embryo showing highly restricted *Wnt9b* expression domain in the facial ectoderm of the maxillary and mandibular processes. (G) Frontal section of an E12.5 embryo showing decreased *Wnt9b* mRNA expression in the facial ectoderm (arrowhead) and the absence of *Wnt9b* expression in the secondary palate. (H) Sagittal section of an E13.5 embryo shows that *Wnt9b* mRNA is expressed at low but detectable levels in the facial ectoderm (arrowhead) at this stage. im, intermaxillary segment of the medial nasal processes; lnp, lateral nasal process; man, mandibular process; max, maxillary process; mnp, medial nasal process; p, palatal shelf; t, tongue.

Fig. 3.

TOPGAL reporter gene expression during facial morphogenesis. (A–C) Lateral view of whole mount E9.5 (A), E10.5 (B) and E11.5 (C) hemizygous transgenic mouse embryos stained with X-gal. The *TOPGAL* expressing tissues are stained green/blue. (A) At E9.5, *TOPGAL* expression is detected in the migrating neural crest cells and cranial ganglia (arrows). A few cells in the distal mandibular arch are also *TOPGAL*-positive. (B, D) Lateral and facial views of E10.5 embryos show *TOPGAL* expression in the distal regions of maxillary, mandibular, medial nasal and lateral nasal processes. (C, E) At E11.5, *TOPGAL* is still expressed in the distal maxillary, medial nasal and lateral nasal processes as these processes fuse to form the upper lip. Expression in the mandibular process is restricted to the rostromedial region adjacent to the median groove. (F) At E12.5, as lip fusion is completed, *TOPGAL* expression is activated in the newly formed vibrissae (arrowheads). Expression in the mandible is significantly down regulated. (G) Frontal section of an E10.5 embryo showing *TOPGAL* expression in the lateral and medial nasal ectoderm, as well as in the limb bud and lung bud. (H) Frontal section of the ventral region of the medial and lateral nasal processes showing *TOPGAL* expression in the pre-fusion ectoderm and underlying

mesenchyme of both nasal processes. Arrow points to expression in the dorsal telencephalon. (I, J) Frontal section of an E11.5 embryo face during the fusion between the medial and lateral nasal processes. J is a higher magnification view of the fusing medial and lateral nasal processes in I. *TOPGAL* is expressed strongly in the epithelial seam (arrowheads) between the nasal processes. Expression is also detected in the mesenchyme directly beneath the expressing ectoderm. Arrow points to *TOPGAL* expression domain in the dorsal telencephalon. ba2, second branchial arch; fl, forelimb bud; hl, hindlimb bud; lb, lung bud; lnp, lateral nasal process; man, mandibular process; max, maxillary process; mnp, medial nasal process.