Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models

Kurt Reynolds1,2,3,*, Priyanka Kumar1,2,*, Lessly Sepulveda Rincon1,2,*, Ran Gu1,2, Yu Ji1,2,3, Santosh Kumar1,2 and Chengji J. Zhou1,2,3,‡

ABSTRACT

Diverse signaling cues and attendant proteins work together during organogenesis, including craniofacial development. Lip and palate formation starts as early as the fourth week of gestation in humans or embryonic day 9.5 in mice. Disruptions in these early events may cause serious consequences, such as orofacial clefts, mainly cleft lip and/or cleft palate. Morphogenetic Wnt signaling, along with other signaling pathways and transcription regulation mechanisms, plays crucial roles during embryonic development, yet the signaling mechanisms and interactions in lip and palate formation and fusion remain poorly understood. Various Wnt signaling and related genes have been associated with orofacial clefts. This Review discusses the role of Wnt signaling and its crosstalk with cell adhesion molecules, transcription factors, epigenetic regulators and other morphogenetic signaling pathways, including the Bmp, Fgf, Tgfβ, Shh and retinoic acid pathways, in orofacial clefts in humans and animal models, which may provide a better understanding of these disorders and could be applied towards prevention and treatments.

KEY WORDS: Orofacial clefts, Cleft lip, Cleft palate, Wnt, Bmp, Fgf, Tgfβ, Shh, Retinoic acid, Epigenetics, Crosstalk

Introduction

Orofacial clefts, mainly cleft lip and/or cleft palate, are among the commonest structural birth defects (Tolarova and Cervenka, 1998; Mossey et al., 2009; Shikoukani et al., 2014; Roosenboom et al., 2015; Kousa and Schutte, 2016). The occurrence of orofacial clefts varies with geographic and ethnic background and with socioeconomic status, with an average rate of 1 in 700 newborns or a range of 0.5-2.6 per 1000 live births (Vanderas, 1987; Croen et al., 1998; Carmichael et al., 2003; Panamonta et al., 2015). Children born with orofacial clefts have severe feeding problems, speech difficulties, frequent middle ear infections and dental defects (Mossey et al., 2009). The long-term and multidisciplinary treatments for these problems are a heavy burden for patients and the healthcare system. Orofacial clefts can either be syndromic or non-syndromic, sporadic or familial (see Glossary, Box 1), and their etiology involves a combination of genetic and environmental risk factors (Tolarova and Cervenka, 1998; Mossey et al., 2009). To date, more than 100 genes have been associated with orofacial clefts (Gritli-Linde, 2007; Juniloff and Harris, 2008; Bush and Jiang, 2012; Iwata et al., 2012), but the underlying mechanisms of these associations remain poorly understood. Mutant mouse models have provided a powerful tool to examine the roles of various genes in contributing to orofacial clefts.

Murine and human facial formation follow a similar developmental trajectory, and facial structures arise from several primordial tissues as described below (Francis-West et al., 1998; Schutte and Murray, 1999; Jiang et al., 2006; Szabo-Rogers et al., 2010; Suzuki et al., 2016). Facial primordia begin to form as early as the fourth week of gestation in humans or embryonic day (E) 9.5 in mice, following the migration of cranial neural crest cells into the frontonasal prominence, paired maxillary prominences (Box 1) and paired mandibular prominences (Cordero et al., 2011). By the fifth week, the medial and lateral nasal prominences (Box 1) outgrow rapidly on either side of the nasal pit. At the ventral junction region, these nasal prominences will subsequently fuse with the maxillary prominence to establish the upper jaw structures, including the upper lip, primary palate (Box 1) and nose. Disruption of any of these craniofaciogenic processes may result in cleft lip with or without cleft palate (CLP). Secondary palate (Box 1) formation is a multifaceted process involving a shift in growth orientation by the palatal shelves (Box 1) (Lough et al., 2017).

In mice, the palatal shelves first emerge from the maxillary prominences at E11.5 and continue to proliferate, elongating ventrally between E12 and E14 (Bush and Jiang, 2012). The elongating palatal shelves consist of mesenchymal tissue with an external epithelial layer. Epithelial-mesenchymal interactions (EMIs) allow communication between the two layers and are important for cell growth and differentiation during many craniofacial developmental processes, including facilitating epithelial-mesenchymal transition (EMT; Box 1) within the palatal shelves during palatogenesis (Sun et al., 1998; Lan and Jiang, 2009; Levi et al., 2011; Santosh and Jones, 2014). The palatal shelves then elevate and continue to grow horizontally toward the midline, which entails significant extracellular matrix remodeling (Bush and Jiang, 2012), until they fuse along the medial edge epithelium (MEE; Box 1) at E14.5-E15. The palatal shelves at the midline fuse both anteriorly and posteriorly from the initial point of contact in a zipper-like manner to form a midline epithelial seam (MES; Box 1). Disintegration of the MES, which may involve apoptosis, EMT and cell migration, is required to establish palatal confluence (Bush and Jiang, 2012). At E15.5-E16.5, the palatal shelves fuse with the nasal septum and the primary palate, separating the nasal and oral cavities, which are required for breathing and feeding after birth (Gritli-Linde, 2007). Disruptions during any stage of palatogenesis can result in a cleft palate (Dixon...
Box 1. Glossary

C6 motif: the six-amino-acid C-terminal domain of Axin proteins. It is implicated in JNK activation, but has no effect on Wnt signaling.

Epithelial-mesenchymal transition (EMT): the induction of adhesive epithelial cells to become migratory and proliferative cells during developmental processes, including in palatogenesis.

Goltz-Gorlin syndrome: a rare genetic disorder, also known as focal dermal hypoplasia (FDH), characterized by distinctive skin abnormalities, including CLP in some cases, and other defects that affect eyes, teeth, and the skeletal, urinary, gastrointestinal, cardiovascular, and central nervous systems. Mutations in PORCN, an upstream regulator of Wnt signaling, are associated with FDH.

Maxillary prominences: a pair of developmental structures at the lateral edges of the oral cavity that give rise to the upper jaw elements, including the maxillary bone.

Medial edge epithelium (MEE): the distalmost edge of the palatal epithelium that surrounds the proliferating mesenchyme. On each palatal shelf, this layer will meet and fuse during secondary palatogenesis.

Midline epithelial seam (MES): the layer of epithelial cells that separates the two lateral pools of the mesenchyme after palatal shelf fusion. MES cells undergo apoptosis to allow the formation of a continuous mesenchyme layer across the secondary palate.

Nasal prominences: two pairs of medial and lateral extensions derived from the unpaired frontonasal prominence during early craniofacial development, which fuse on either side with the maxillary prominences to form the primary palate and nostrils, and separate the nasal cavity from the oral cavity.

Neurulation: a stage in vertebrate embryogenesis in which the neural plate folds to form the neural tube.

Palatal shelf: a pair of palatal structures elongated from the maxillary prominences between the nasal prominence and the first branchial arch, resulting in the fusion of the left and right palatal shelves posterior to the primary palate.

Primary palate: a rostralmost palatal structure formed by the fusion of the nasal and maxillary prominences to separate the nasal pits from the oral cavity.

Regulator of G protein signaling (RGS) domain: a motif required for the protein’s activity in accelerating the GTPase activity of G-proteins. The RGS domain in Axin proteins is required for binding APC in Wnt signaling.

Robinow syndrome: congenital syndrome characterized by craniofacial, skeletal and urogenital defects, which frequently includes orofacial clefts and has been associated with mutations in non-canonical Wnt signaling genes, including WNT5A and ROR2.

Rugae: the series of ridges produced by folding of the anterior wall of the palate behind the incisive papillae.

Secondary palate: a roof structure of the oral cavity that arises from the fusion of the left and right palatal shelves posterior to the primary palate.

Sporadic or familial CLP: occurrence of CLP within families or close relatives is referred to as familial CLP, whereas appearance of the same phenotype in humans and mouse models (Mani et al., 2010), and includes multiple distinct pathways that are activated by the binding of the secreted Wnt ligand proteins to a complex receptor system. Wnts bind to the frizzled (Fzd) receptors along with the co-receptors, such as members of the low-density lipoprotein receptor-related protein (Lrp) or receptor tyrosine kinase-like orphan receptor (Ror) families, at the surface of the Wnt-responding cells (Fig. 1, Box 2). The ligand-receptor complex interacts with cytoplasmic proteins, such as the axis inhibition (Axin) and disheveled (Dvl) proteins, to trigger intracellular signaling (Wallingford and Basler, 2005; Niehrs, 2012; Stamos and Weis, 2013; Bernatik et al., 2011) (Fig. 1, Box 2). Wnt pathways are broadly classified as β-catenin-dependent canonical and β-catenin-independent non-canonical pathways, such as the planar cell polarity (PCP) pathway (Box 2) and the Wnt/Ca2+ pathway (Komiya and Habas, 2008; Gao and Chen, 2010). This Review discusses the role of Wnt signaling and its crosstalk with other signaling pathways in orofacial cleft etiology and related developmental processes, which may provide a better understanding of basic mechanisms and future translational applications.

Wnt signaling genes associated with orofacial clefts in humans

Both syndromic and non-syndromic orofacial clefts have been attributed to mutations of various Wnt signaling component genes (Table 1). Nascent WNT proteins are lipid modified by the enzyme porcupine O-acyltransferase (PORCN) within the endoplasmic reticulum of the WNT-producing cell and subsequently transported by WNT secretory mediator (WLS, also known as GPR177) through the Golgi apparatus to the cell surface for secretion (Port and Basler, 2010; Barrott et al., 2011) (Fig. 1, Table 1, Box 2). An extensive number of mutations throughout the coding region and large gene rearrangements of PORCN have been identified in focal dermal hypoplasia or Goltz-Gorlin syndrome (Box 1), which includes orofacial clefts (Table 1) (Lombardi et al., 2011). However, a role for PORCN in non-syndromic cleft lip and palate (NSCLP), or a role for WLS in human orofacial clefts, has not been demonstrated. A homozygous nonsense mutation in WNT3 has been correlated with orofacial clefts and tetra-amelia syndrome (Box 1) (Niemann et al., 2004). Meanwhile, multiple non-coding single-nucleotide polymorphisms (SNPs) in WNT3 have been associated with NSCLP in a wide range of populations, including Latin American, European and Chinese (Chiquet et al., 2008; Nikopensius et al., 2010; Nikopensius et al., 2011; Mostowska et al., 2012; Lu et al., 2015). Yet, in some populations, such as Caucasian Brazilian, the relationship between WNT3 variants and NSCLP remains unclear (Fontoura et al., 2015; Machado et al., 2016). Intriguingly, Nikopensius et al. (2010) reported a potential epistatic interaction between WNT3 and collagen, type II, alpha 1 (COL2A1), an important gene in the production of collagen. Mutations in either et al., 2011). Although the mechanisms that drive palatogenesis are believed to be conserved among mammals, differences in the morphological structures, and in the interactions that occur during palatal closure, exist between species (Yu et al., 2017). An extensive list of different mouse models for cleft lip and/or cleft palate has been previously reviewed elsewhere (Gritli-Linde, 2007; Gritli-Linde, 2008; Juriloff and Harris, 2008; Funato et al., 2015). However, mutations in specific genes do not always produce the same phenotype in humans and mouse models (Gritli-Linde, 2008).
Box 2. Wnt signaling

Wnts are secreted lipid-modified signaling proteins that are evolutionarily conserved and play vital roles in development, homeostasis and disease. Nineteen Wnt ligand proteins encoded by respective genes in mammalian genomes act through a variety of receptors and co-receptors, including ten seven-transmembrane frizzled (Fzd) receptors, two single-transmembrane co-receptors Lrp5/6, and the receptor tyrosine kinase-like receptors Ror1/2 and Ryk.

In the Wnt-producing cells, the nascent Wnt proteins are palmitoylated by porcine O-acyltransferase (Porcn), followed by their secretion in the extracellular matrix via Wnt ligand secretion mediator (Wls; Fig. 1). Wnt signaling is initiated when a secreted Wnt ligand binds to a Fzd receptor along with a Lrp co-receptor in the canonical pathway, or to a tyrosine kinase-like Ror or Ryk receptor in the non-canonical pathway. The ligand-receptor interaction at the surface of the Wnt-responding cell is modulated by various positive or negative regulatory factors and is transmitted through numerous intracellular molecules. Three major signaling pathways have been demonstrated downstream of the initial Wnt ligand-receptor interaction: the canonical Wnt/β-catenin signaling pathway, the non-canonical planar cell polarity (PCP) pathway, and the Wnt/Ca²⁺ pathway, which is less understood.

Canonical Wnt/β-catenin pathway: when Wnts are absent, intracellular β-catenin is constantly phosphorylated for degradation by the glycogen synthase kinase Gsk3β in the β-catenin destruction complex, which also includes the tumor suppressing Axin proteins and adenosomatous polyposis coli (APC), the casein kinase CK1, the protein phosphatase 2A (PP2A) and the E3-ubiquitin ligase β-TrCP. Upon the binding of a Wnt ligand to a Fzd receptor, the Fzd recruits a Dvl cytoplasmic phosphoprotein and a Lrp co-receptor recruits an Axin, which inhibits the destruction complex. This stabilizes cytoplasmic β-catenin, resulting in its accumulation and translocation into the nucleus. There it binds to Tcf/Lef1 transcription factors to regulate the transcriptional activation of critical Wnt target genes in various cells/tissues, such as the orofacial cleft-associated genes Msi1/Msx2 in orofacial primordia (Fig. 1).

Non-canonical PCP pathway: The binding of a Wnt ligand to Ror or Ryk receptors promotes the interaction of a Dvl with diseveled-associated activator of morphogenesis 1 (Dapper1), which activates several downstream GTPases, including the Rac proteins and ras homolog family member A (Rhoa). This results in the restructuring of actin to change cell shape, polarity and movement. Dvl can also activate phospholipase C to generate inositol trisphosphate, which activates the release of Ca²⁺ to trigger a number of downstream effects, such as cell migration and proliferation.

gene are associated with NSCLP (Nikopensius et al., 2010; Nikopensius et al., 2011), and COL2A1 mutations also cause Stickler Syndrome, which frequently includes a cleft palate, is associated with mutations in WNT4 (Person et al., 2010), along with mutations in the co-receptor gene receptor tyrosine kinase-like orphan receptor 2 (ROR2) (Afzal et al., 2000; van Bokhoven et al., 2000) and the signal transducer DVL1 (Bunn et al., 2015; White et al., 2015), indicating the importance of a non-canonical WNT5A/ROR2/DVL1 signaling cascade in human patogenesis. A missense mutation and rare haplotypes of another non-canonical co-receptor gene, receptor-like tyrosine kinase (RYK), have also been linked to NSCLP in Vietnamese and Japanese patients (Watanabe et al., 2006).

Analysis of an African American family with 11 members displaying NSCLP identified a variant of the WNT receptor gene FZD6 with an intronic mutation that creates a protein-binding site, resulting in decreased expression and contributing to CLP (Cvjetkovic et al., 2015). Among other FZD genes, a nonsense mutation of FZD2 was identified in a family with omodylosplasia that includes CLP (Saal et al., 2015). By contrast, frameshift, nonsense and missense mutations in the WNT co-receptor gene LRPs have been associated with orofacial clefts and tooth agenesis (Basha et al., 2018; Ockeloen et al., 2016), suggesting that deficient LRP6-mediated canonical WNT signaling has a crucial role in CLP pathogenesis. However, de novo nonsense and frameshift mutations in the key canonical WNT signaling mediator gene catenin beta 1 (CTNNB1; encoding β-catenin) were linked with abnormal craniofacial features, but not with orofacial clefts (Tucci et al., 2014). Conversely, analysis of variants of the β-catenin destruction complex genes AXIN2 and glycogen synthase kinase 3 beta (GSK3B) in NSCLP families across multiple populations identified intronic SNPs that contribute to orofacial clefts (Leta et al., 2009; Letra et al., 2012; Vijayan et al., 2018) (Table 1), suggesting that excessive WNT/β-catenin signaling also contributes to CLP pathogenesis. Nevertheless, gene association studies in humans with orofacial clefts have proven challenging, complicated by the fact that the same variants can be associated with orofacial clefts in one population but not in others. Therefore, animal models, especially the mutant mouse model, have a crucial role in investigating the genetic mechanisms of orofacial clefts in mammals.

Wnt signaling genes as the cause of orofacial clefts in animal models

Mutations in various Wnt signaling genes cause orofacial clefts in animal models (He and Chen, 2012) (Table 1). The following discussion highlights animal models of orofacial clefts involving mutations in both canonical and non-canonical Wnt signaling components, from ligand secretion through signal transduction, focusing predominantly on mouse models.

Mouse models with mutations in regulatory genes upstream of Wnt

Absence of Porcn or Wls from Wnt-producing cells (Fig. 1) results in Wnt protein retention, which leads to Wnt signaling failure (Barrott et al., 2011). Conditional ablation of Porcn in neural crest cells results in defective facial formation in mice, including CLP (Bankhead et al., 2015) (Fig. 1). Previous studies suggest that Wls is
required for Wnt/β-catenin signaling during craniofacial development (Fu et al., 2011). Facial ectodermal/epithelial ablation of either β-catenin or Wls arrests the formation of orofacial primordia (Wang et al., 2011; Zhu et al., 2016), and conditional knockout of Wls in craniofacial neural crest cells with a Wnt1-driven Cre recombinase causes cleft palate (Liu et al., 2015) (Table 1). Additionally, Rspo2, a member of the R-spondin family, is a well-known enhancer of canonical Wnt signaling (Fig. 1). Rspo2 loss-of-function mice exhibit cleft palate with a partially penetrant cleft lip, along with mandibular hypoplasia and maxillary and mandibular skeletal deformation, which are caused by defective patterning and morphogenesis of the first pharyngeal arch due to altered EMI (Yamada et al., 2009; Jin et al., 2011). However, cleft palate in Rspo2-null mice is likely caused by delayed palatal shelf (Box 1) elevation, a possible secondary effect of aberrant mandible and tongue morphogenesis (Jin et al., 2011). Nevertheless, Wnt signaling may mediate Tgfβ signaling to regulate EMI in muscle development of the soft palate (Iwata et al., 2014).
Table 1. Summary of WNT signaling genes associated with orofacial clefts in humans and animal models

| Gene     | Role                                      | Mutations and human association                                                                 | Animal model                                                                 | Signaling mechanism                                                                 | References                                                                 |
|----------|-------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| PORCN    | Aclyltransferase that modifies nascent WNT proteins | At least 68 coding mutations and 12 large gene rearrangements found in focal dermal hypoplasia or Goltz–Gorlin syndrome, which can include CLP | Cre recombinase ablation in ectodermally derived tissues results in CLP         | Loss of Porcn in oral ectoderm prevents Wnt signaling efficacy                      | Lombardi et al., 2011; Herr and Basler, 2012; Bankhead et al., 2015      |
| WLS      | WNT ligand transportation and secretion    | No association study reported                                                                  | Conditional knockout with Wnt1-Cre resulted in brain and craniofacial defects including cleft palate | Upregulation of Mx1; upregulation of Shh in the anterior palate; Wls may act through Wnt5a in the palatogenesis | Fu et al., 2011; Liu et al., 2015                                        |
| WNT3     | Ligand                                    | Syndromic and non-syndromic CLP in European and Chinese populations                             | Wnt3-null mutant mouse embryos die around E10.5, before lip/palate formation  | Epistatic interaction with COL2A1, as well as interactions with FGFR1 and MTHFR genes in humans | Liu et al., 1999; Niemann et al., 2004; Chiquet et al., 2008; Menezes et al., 2010; Nikopensius et al., 2010; Nikopensius et al., 2011; Lu et al., 2015 |
| WNT3A    | Ligand                                    | Five non-coding SNPs and four haplotypes with NSCLP in European American and Hispanic populations; intronic and 3’ UTR variants rs3121310 and rs752107 in a Chinese cohort | Knockout mice die around E12.5, prior to palatogenesis. Conditional ablation data unavailable | Wnt3a interacts with Fzd2/ Fzd7 and provides synergistic effects in the manifestation of a cleft palate phenotype | Chiquet et al., 2008; Luis et al., 2009; Yao et al., 2011; Mostowska et al., 2012; Yu et al., 2012 |
| WNT5A    | Ligand                                    | Non-coding SNP rs566926 with NSCLP and unilateral CLP in European American, Hispanic and Caucasian Brazilian populations; interaction with WNT3A in Hispanic population | Knockout mice display fully penetrant cleft palate with limb and tail defects, and downregulation of Bmp2, Bmp4, Shh, PchI, Msx1 | Wnt5a lies at the interface of several signaling pathways; the Wnt5a/ CaMKII pathway induces RA signaling during palate development | Yamaguchi et al., 1999; Chiquet et al., 2008; He et al., 2008; Menezes et al., 2010; Menezes et al., 2012; Cong et al., 2014 |
| WNT6     | Ligand                                    | Clustered with WNT7A and SNPs among CLP trios from three populations [Maryland (USA), Singapore and Taiwan] | Wnt6 regulates the viability of MEPM cells; in vivo loss of function data unavailable | Wnt6 is involved in palatogenesis through its activation of the j-catenin pathway | Beaty et al., 2006; Jiang et al., 2017                                    |
| WNT7A    | Ligand                                    | Missense mutation c.1019G>A (p.S340N) with NSCLP in Columbian families                         | Craniofacial phenotype not described in Wnt7a LOF mice.                      | Wnt7a acts through Fzd10                                                             | Nunnally and Parr, 2004; Pengelly et al., 2016                           |
| WNT8A    | Ligand                                    | Haplotype with NSCLP in European American and Hispanic populations                             | Craniofacial phenotype not described in Wnt8a LOF mice.                     | Wnt8a might interact with Bmp4 and Notch signaling during embryogenesis            | Chiquet et al., 2008; Castro Colabianchi et al., 2018                    |
| WNT9B    | Ligand                                    | Intronics rs1530364 and rs1105127 SNP variants with NSCLP in a northeastern European population | Incomplete penetrance of CLP in Wnt9b-null mouse embryos; full cleft lip penetration when doubly knocked out with Pbx1  | Possible epistatic interaction with MSX1 predicted in human populations; Pbx1 may act upstream; Wnt4, Fgf8, Msx1, Max2, Axin2 are downstream | Carroll et al., 2005; Junlioff et al., 2006; Ferretti et al., 2011; Nikopensius et al., 2011; Jin et al., 2012 |
| WNT10A   | Ligand                                    | Clustered with WNT7A and SNPs among CLP trios; c.392C>T (A131 V) substitution with NSCLP in a northeastern Chinese population | Short tibia and oligodontia in conditional deletion of Wnt10a mice, no CLP phenotype is observed | Wnt10a knockout inhibits cell proliferation and increases apoptosis via Wnt/β-catenin signaling pathway | Beaty et al., 2006; Feng et al., 2013; Feng et al., 2014; Xu et al., 2017 |
| WNT11    | Ligand                                    | Non-coding SNPs linked to NSCLP in European American populations                             | Altered Wnt11 expression identified in a mouse strain with spontaneous CLP. Wnt11 knockout in culture prevents palate fusion | Wnt11-dependent apoptosis is required for the fusion of palatal shelves            | Chiquet et al., 2008; Lee et al., 2008; Sasaki et al., 2014              |
Table 1. Continued

| Gene     | Role                        | Mutations and human association                                      | Animal model                                                                 | Signaling mechanism                                   | References                                                                 |
|----------|-----------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------------------------|
| **FZD1** | WNT receptor                | No association with NSCLP                                               | Double knockout with Fzd2 resulted in fully penetrant CPO with cardiac defects | Function redundantly with Fzd2                           | Yu et al., 2010; Wang et al., 2012a                                       |
| **FZD2** | WNT receptor                | A nonsense mutation (c.1644G>A; p.W548*) in a family with omodysplasia including CLP | Homozygous knockout mice have 50% penetrant CPO. Double knockouts with Fzd1 have fully penetrant CPO with cardiac defects | Required for Wnt signal transduction in craniofacial development; function redundantly with Fzd1 | Yu et al., 2010; Saal et al., 2015                                          |
| **FZD6** | WNT receptor                | A rare variant in intron 1 of FZD6 (rs138557689) linked to NSCLP in an African American family | No craniofacial phenotypes described in Fzd6 knockout mice                     | May interact with RA signaling and its expression becomes enhanced in embryonic stem cells upon RA treatment | Osei-Sarfo and Gudas, 2014; Cvjetkovic et al., 2015                         |
| **LRP6** | WNT co-receptor             | Frameshift (p.A383G fs*8, p.L742F fs*7, p.C1532 fs), nonsense (p.E594*, p.R1125*) and missense (p.A19V) mutations with orofacial clefts and tooth agenesis | Lrp6 knockout mice display fully penetrant CLP                                | Activates Msx1/Max2 in orofacial mesenchymal cells and Sostdc1 in the surface ectoderm; represses Aid1 in the upper lip primordia | Basha et al., 2018; Massink et al., 2015; Ockeloen et al., 2016; Song et al., 2009 |
| **ROR2** | WNT co-receptor             | SNPs in 5’UTR (rs7858435) and intron (rs10820914) linked to NSCPO in Asian populations | Ror2 knockout results in cleft palate with vertebral defects                   | Interaction with Wnt5a as upstream regulator and Dvl as downstream effector | Oishi et al., 2003; Schwabe et al., 2004; He et al., 2008; Ho et al., 2012; Wang et al., 2012b |
| **RYK**  | WNT co-receptor             | A missense mutation 1355G>A (Y452C) and rare haplotypes linked to NSCLP in Vietnamese and Japanese patients | Ryk knockout results in cleft palate with craniofacial and limb defects in mice | Crosstalk with Ephb2/3                                      | Halford et al., 2000; Watanabe et al., 2006                                |
| **RSPO2**| WNT/LRP6 signaling amplifier| Nonsense and frameshift mutations (p.Q70*, p.E137*, p.G42V fs*49) linked to Tetra-amelia syndrome with CLP | Rspo2 knockout yields limb and craniofacial defects with 71% cleft secondary palate without cleft lip. The incidence of cleft lip is less than 10% in Rsopo2 mutant mice | Downregulation of Axin2, Wnt3 and Sp8 genes in the Rspo2-null orofacial primordia | Nam et al., 2007; Jin et al., 2011; Jiang et al., 2017; Szenker-Ravi et al., 2018 |
| **CTNNB1**| Encodes β-catenin, which mediates canonical WNT signaling transcriptional activation and cell adhesion in conjunction with CDH1 | De novo nonsense and frameshift mutations (p.Q309*, p.S42ST fs*11, p.R515*, and p.G236R fs*35) cause characteristic abnormal craniofacial features without orofacial clefts | Epithelial β-catenin ablation or constitutive activation results in highly penetrant cleft palate | Altered expression of Axin2 and Tgfβ3 observed in the palatal shelves of β-catenin mutant mice | He et al., 2011; Tucci et al., 2014                                       |
| **AXIN1**| Scaffold for β-catenin destruction complex | No significant association found in Polish NSCLP patients               | Axin1 mutant mice display cleft palate with midfacial cleft                  | Axin1 is a key modulator of β-catenin activity in mouse craniofacial development | Chia et al., 2009; Mostowska et al., 2012                                  |
| **AXIN2**| Scaffold for β-catenin destruction complex | Intronic (rs7224837, rs3923086), synonymous (rs2240307), missense (rs2240308) and 5’UTR (rs7591) mutations linked to NSCLP in Caucasian and Hispanic families, as well as in Chinese Han and Brazilian populations | Craniofacial defects observed in Axin2-knockout mice. | Axin2 seems to be a key modulator of β-catenin activity during human craniofacial development | Yu et al., 2005b; Letra et al., 2009; Letra et al., 2012; Mostowska et al., 2012; Han et al., 2014 |
| **GSK3B**| Kinase that targets β-catenin for destruction | Intronic SNP (rs13314595) associated with NSCLP in Caucasian population | Cleft palate and midline defects in knockout mice                            | Upregulates β-catenin, Axin2 and Wnt9b, and downregulates Ihh, Shh, Ptc1 and Gli1, in the mutants; Dkk1 treatment rescues Gsk3β expression in vitro | Liu et al., 2007; Nelson et al., 2011; Vijayan et al., 2018                |

Continued
Table 1. Continued

| Gene | Role | Mutations and human association | Animal model | Signaling mechanism | References |
|------|------|--------------------------------|--------------|-------------------|------------|
| **LEF1** | Transcription factor | Intronic SNPs (rs10022956 and rs10025431) linked to NSCLP in an Italian cohort | Canonical Wnt signaling via Lef1 is required for tooth development in mice | Lef1 activation by Tgfl3 induces EMT in the mouse palate; Lef1 in the dental epithelium also activates Fgf signaling | Kratochwil et al., 2002; Nawashad and Hay, 2003; Martinelli et al., 2011a |
| **PRICKLE1** | Nuclear receptor involved in the PCP pathway | Two rare missense mutations c.1138C>T (p.L380F), c.2026C>T (p.R676W), and one SNP (rs12658) in 3’ UTR associated with incompletely penetrant NSCLP in the Philippine population | Null and hypomorph Prickle1 mutant mice phenocopy human Robinow syndrome with cleft palate | Prickle1 is a proteasomal target of Wnt5a signaling; Dvl2 is misregulated in Prickle1 mutants | Liu et al., 2014a; Yang et al., 2014 |

**Mouse models with mutations in canonical Wnt signaling genes**

Among the 19 Wnt ligands, Wnt2, Wnt2b, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, Wnt9a, Wnt10a, Wnt10b, Wnt11 and Wnt16 are expressed in the palatal primordia during palatogenesis (Warner et al., 2009). Five Wnts appear to be temporally regulated in embryonic palatal tissue, showing more than 2.0-fold changes in expression levels, either between E12.5 and E13.5, or between E13.5 and E14.5. Of these five ligands, Wnt4, Wnt10a and Wnt10b are expressed in epithelial tissues, while Wnt2 and Wnt16 are expressed in the mesenchyme (Warner et al., 2009). However, the roles of these temporarily expressed Wnts in palatogenesis remain unclear. By contrast, Wnt6 has been demonstrated to play a role in palatal shelf elongation and elevation through the activation of the β-catenin pathway, promoting cell proliferation in the palatal mesenchyme (Jiang et al., 2017) (Table 1).

Wnt9b might activate the canonical Wnt signaling pathway during midfacial development (Lan et al., 2006). Wnt9b-null mice die perinatally, exhibiting incompletely penetrant CLP (Carroll et al., 2005; Juriloff et al., 2006; Ferretti et al., 2011) (Table 1, Fig. 1), while ablation of Wnt9b in the facial ectoderm also causes CLP (Jin et al., 2014a). These findings suggest a key role of facial ectodermal and epithelial Wnt/β-catenin signaling in primary lip and palate formation and fusion. In addition, Wnt3 may also regulate midfacial development, as well as lip fusion, through the canonical Wnt pathway, with both Wnt9b and Wnt3 playing distinct roles during midfacial morphogenesis (Lan et al., 2006). Wnt3-null embryos do not survive beyond E10.5, while morphological differences from wild-type embryos become apparent from E6.5 onward (Liu et al., 1999). *In vitro* experiments further suggest that Wnt3 and Wnt9b may activate canonical Wnt signaling during palatogenesis through the receptors Fzd1 and Fzd2 (Huang et al., 2006; Yu et al., 2010). Palatal shelves fail to close in doubly homozygous Fzd1 and Fzd2 knockout mice with complete penetrance (Yu et al., 2010) (Table 1, Fig. 1), while Fzd7 is highly redundant with Fzd2 during palatogenesis (Yu et al., 2012). Canonical Wnt signaling through the co-receptor Lrp6 plays an indispensable role in primary lip and palate formation and fusion (Song et al., 2009; Zhou et al., 2010). Lrp6-deficient mutant mouse embryos exhibit fully penetrant CLP as a consequence of diminished Wnt signaling and disrupted expression of downstream target genes in the orofacial primordia (Song et al., 2009) (Fig. 1).

While conditional loss of function of β-catenin in palatal epithelial cells leads to cleft palate, conditional gain of function of β-catenin in the epithelium also leads to cleft palate and aberrant fusion between the palate shelf and mandible (He et al., 2011), suggesting crucial roles of epithelial Wnt signaling in palatal shelf fusion. Moreover, homozygous knockout of Gsk3b, which encodes a β-catenin-degrading enzyme in the canonical Wnt signaling pathway, results in mice displaying full cleft palate (Liu et al., 2007) (Table 1, Fig. 1), suggesting that excessive β-catenin signaling also causes cleft palate in these mouse models.

Axin1 is another component of the β-catenin destruction complex and therefore a negative regulator of Wnt signaling (Fig. 1). Early embryonic lethality is observed in homozygous Axin1 mutant mouse embryos carrying alleles with deletions in either the regulator of G protein signaling (RGS) domain (Box 1) or the C6 motif (Box 1) that encodes the six C-terminal amino acids (Axin1<sup>C6</sup>) (Chia et al., 2009). Intriguingly, many mouse embryos with compound mutant alleles of *Axin1<sup>ΔC6</sup>* and *Ctnnb1<sup>ΔΔC</sup>* can survive to term but develop craniofacial defects, including CLP (Chia et al., 2009) (Table 1, Fig. 1). This suggests that diminished Wnt/β-catenin signaling can partially rescue the early lethality that is likely caused by excessive β-catenin signaling, but it cannot rescue the CLP phenotype that may be caused by both excessive β-catenin and defective JNK signaling (Chia et al., 2009). Together, these findings highlight the importance of appropriate spatiotemporal control of Wnt/β-catenin signaling and the complexity of the regulatory processes in lip and palate development.

**Mouse models with mutations in non-canonical Wnt signaling genes**

Wnt5a acts through the non-canonical Wnt pathway to alter directional cell movements (Liu et al., 2015). Wnt5a-null mouse embryos exhibit cleft palate (Table 1, Fig. 1), along with other phenotypes, such as defective outgrowth of the snout, tongue, mandible, limb, tail and other skeletal defects, leading to perinatal lethality (Yamaguchi et al., 1999; Li et al., 2002; Yang et al., 2003; Cervantes et al., 2009; Tai et al., 2009; Buttler et al., 2013; Okamoto et al., 2014). Wnt5a plays a key role in the migration of mesenchymal cells during palatogenesis (Xiao et al., 2005; He et al., 2008), possibly acting through Ror2, which is expressed in the mesenchyme of the secondary palate (Schwabe et al., 2004). Studies suggested that Wnt5a binds to the cysteine-rich domain of Ror2 to activate the non-canonical Wnt pathway, interacting both physically and functionally (Oishi et al., 2003). In mesenchymal cell culture, cell migration seems to be driven by the Wnt5a-Ror2-Kif26b signaling cascade (Susman et al., 2017), further suggesting the
significance of this non-canonical Wnt signaling cascade in palatogenesis. Furthermore, phosphorylation of the Wnt signal transducer Dvl2 seems to be triggered by the Wnt5a-Ror2 pathway, and Dvl2 may be the molecular switch that allows Wnt5a to activate both non-canonical and canonical Wnt pathways (Ho et al., 2012).

Ror2 knockout mice display craniofacial defects, including cleft palate, further implicating this cascade in the etiology of non-canonical Wnt-signaling-caused orofacial clefts (Schwabe et al., 2004). It has also been suggested that the Ryk receptor may interact with Ror2 to bind Wnt5a (Oishi et al., 2003), and mutations in Ryk also cause cleft palate in mice (Halford et al., 2000) (Table 1, Fig. 1). In addition, ablation of the non-canonical Wnt signaling molecule Prickle1 causes cleft palate and limb defects (Yang et al., 2014) (Table 1, Fig. 1), which are similar to those of Wnt5a mutants (He et al., 2008). However, Prickle1 mutants present less severe limb defects than Wnt5a mutants, implying that the transduction of Wnt5a signaling might not act through Prickle1 alone. Similarly to Wnt5a mutants, Prickle1 knockout mice present with improper sonic hedgehog (Shh) expression during palatogenesis (Yang et al., 2014). Furthermore, Prickle1 has been shown to act downstream of Wnt5a and interact with Dvl2, and Prickle1 mutants display characteristics that resemble Robinow syndrome (Liu et al., 2014a) (Fig. 1). Thus, a signaling cascade of Wnt5a-Ror2-Prickle1/Dvl2 might be crucial for proper tissue growth and morphogenesis during palatogenesis in mice.

Zebrafish models
Although mouse models have vastly contributed to our understanding of Wnt signaling in palatogenesis, other models, such as the zebrafish, provide unique insight into craniofacial formation and the basic requirements for palate formation (Duncan et al., 2017). Canonical Wnt signaling through Lrp5 is required for appropriate cranial neural crest cell migration, but not their induction, and for craniofacial morphogenesis in zebrafish (Willems et al., 2015). Wnt9a is expressed in the zebrafish pharyngeal arch, implicating its role during craniofacial development (Curtin et al., 2011). Interestingly, Wnt9a has been shown to play a role in palatogenesis in fish, but not in mammals (Dougherty et al., 2013; Rochard et al., 2016), suggesting taxon-specific Wnt signaling functions in palatogenesis. Wnt5b is thought to assume a similar craniofacial role in zebrafish that Wnt5a plays in mammals (Topczewski et al., 2011). Non-canonical Wnt signaling mediated by epithelial Wnt5b and Wnt9b was demonstrated to stimulate the PCP pathway in chondrocytes, facilitated by Secreted frizzled-related protein 3 (Sfrp3, also known as Frzb) and Glypican 4 (Gpc4) activity during palate extension (Rochard et al., 2016). Additionally, morpholino-based knockdown of Wnt3a and Tubulointerstitial nephritis antigen-like 1 (Tinag1), a Wnt-interacting extracellular matrix protein, results in defects of the pharyngeal arch and ethmoid plate, which corresponds to the mammalian palate (Neiswender et al., 2017). Loss of function of the Wnt modulator Sfrp3 in zebrafish results in the failure of anterior palate extension, further highlighting the role of Wnt signaling in palatal extension and convergence in zebrafish (Kamel et al., 2013).

Chick models
Orofacial clefts have also been observed in chick embryos (Abramyan and Richman, 2018), where Wnt signaling similarly mediates the growth of primordial facial processes and the developing palate, in which six epithelial and three mesenchymal Wnt ligands, as well as several other pathway components, are expressed (Geetha-Loganathan et al., 2009). Wnt11 was shown to activate the non-canonical Wnt/PCP pathway and inhibit canonical Wnt/β-catenin signaling in the maxillary prominence, and its ectopic expression results in a notched beak/cleft lip phenotype (Geetha-Loganathan et al., 2014). Similarly, overexpression of Wnt2b leading to ectopic expression of msh homeobox 1 (Msx1) results in a foreshortened rostrum/upper beak, corresponding with a mammalian CLP phenotype (Medio et al., 2012).

Frog models
Recently, the suitability of Xenopus embryos for transplanting tissue and local chemical perturbation have provided a suitable clefting model (Dickinson, 2016). Although several studies have assessed the involvement of various biochemical pathways and factors in frog palatal clefts, including retinoic acid and folate metabolism, few studies have probed Wnt signaling during orofacial development in this organism (Dickinson and Sive, 2009; Kennedy and Dickinson, 2012; Wahl et al., 2015).

In vitro models
Another means by which investigators study secondary palate fusion is by culturing palatal shelf explants and assaying their ability to complete the final stages of palate fusion in vitro, such as adherence and formation of the MES and subsequent apoptosis to establish mesenchymal confluence (Ibrahim et al., 2015). Although not directly analogous to in vivo palatogenesis, this approach has helped examine the roles of many factors and processes that are important for the fusion process specifically, including Wnt11 and its dependence on Fgf signaling in palatal closure (Lee et al., 2008).

Crosstalk between Wnt signaling, cell adhesion molecules and transcription factors in orofacial clefts
Because β-catenin has dual roles in Wnt signaling and in cell adhesion, it remains unclear which functions of β-catenin are required for which stages of orofacial development. The roles of other cell-cell adhesion proteins, such as E-cadherin (Cdh1), during palatogenesis remain to be elucidated (reviewed in Lough et al., 2017). Mutations in CDH1 have been associated with an increased risk for non-syndromic orofacial clefts in humans (Rafighdoost et al., 2013; Vogelaar et al., 2013; Bureau et al., 2014; Hozyasz et al., 2014; Brito et al., 2015; Ittiwut et al., 2016; Song et al., 2017). In mouse models, Cdh1 knockout is embryonic lethal and mutant embryos do not develop beyond E10.5 (García-Higueru et al., 2008). Conditional Cdh1 knockout in neural crest cells results in craniofacial defects related to bone development, including a shortened snout, abnormal teeth and twisted nasal bones (Shao et al., 2016). However, these mutants did not develop orofacial clefts. A possible interaction between Cdh1 and the Wnt signaling pathway has been suggested in human endometrial epithelial cells, where ablation of Cdh1 enhances canonical Wnt signaling (Zhu et al., 2018) (Fig. 2). Furthermore, increased expression of Cdh1 in mouse maxillary mesenchymal cells during palatogenesis results in a reduction of cytosolic β-catenin (Warner et al., 2016) (Table 2). These studies suggest that Cdh1 may negatively regulate the canonical Wnt/β-catenin signaling pathway in humans and mice (Table 2, Fig. 2).

Individuals with mutations in either of the epithelial transcription factors grainyhead-like transcription factor 3 (GRHL3) and interferon regulatory factor 6 (IRF6), detected in families with Van der Woude syndrome (Box 1), tend to present with CLP (de Lima et al., 2009; Peyrard-Janvid et al., 2014). Further investigation in mice suggested that there is no epistatic interaction between these two transcription factors during palatogenesis (Peyrard-Janvid et al., 2014).
epithelium during palatogenesis seems to be crucial for normal palate formation (Carpinelli et al., 2017). In mice, a cooperative interaction has been suggested between Grhl2 and Grhl3 during primary neurulation (Box 1) (Rifat et al., 2010), and Grhl3 might act downstream of canonical Wnt signaling during neural tube closure (Kimura-Yoshida et al., 2015). Nevertheless, it remains unclear whether Grhl3 is a direct downstream target of the canonical Wnt/β-catenin signaling, or whether Grhl2 and Grhl3 act cooperatively during palate development.

Analysis of Irf6 expression and function in mouse and chick developmental models suggests that Irf6 might play a role in tissue fusion events during palatogenesis (Knight et al., 2006; Velazquez-Aragon et al., 2016). The Wnt target p63 (also known as TP63), a key regulator of proliferation and differentiation (Truong et al., 2006), inhibits the Wnt signaling output by repressing Wnt/β-catenin responsive elements in target genes (Katoh et al., 2016). p63 may directly activate Irf6 in the facial ectoderm, and a defective pre-B cell leukemia homeobox (Pbx)-Wnt-p63-Irf6 signaling cascade has been suggested in cleft lip formation (Ferretti et al., 2011). Irf6 interacts upstream of the cleft-associated Rho GTPase-activating protein 29 (Arhgap29), which regulates Rho activity downstream of Wnt5a in the PCP pathway (Leslie et al., 2012). Furthermore, several genome-wide association studies have uncovered interactions between IRF6 and other factors linked with orofacial cleft susceptibility. Li and colleagues have identified a three-way gene interaction of SNPs in IRF6, WNT5A and Clorf107 (also known as UTP25, a nucleolar protein), and a separate separation between IRF6 and WNT7A, in association with NSCLP (Li et al., 2015). IRF6 mutations were also associated with SNPs in the actin-binding protein tropomyosin (TPM1) and in the axon guidance signaling molecule netrin 1 (NTN1) (Velazquez-Aragon et al., 2016).

Wnt signaling crosstalk with other morphogenetic signaling pathways in orofacial clefts

Wnt signaling does not act in isolation during lip/palate development. Several other signaling pathways are involved in orofacial development, including the fibroblast growth factor (Fgf), bone morphogenetic protein (Bmp), transforming growth factor beta (Tgfβ), sonic hedgehog (Shh) and retinoic acid (RA) signaling pathways (Iwata et al., 2011; Bush and Jiang, 2012; Cobourne and Green, 2012; Parada and Chai, 2012; Stanier and Pauws, 2012; Wang et al., 2013; Kurosaka et al., 2014; Okano et al., 2014; Yan et al., 2018; Graif et al., 2016). This section discusses how these pathways interact with each other and with Wnt signaling.

Wnt-Fgf signaling crosstalk

Wnt/β-catenin signaling activates Fgf8 expression in early facial patterning (Wang et al., 2011), while Fgf8 induces paired box 9 (Pax9) expression during palatogenesis (Neubuser et al., 1997) (Fig. 2). Fgf8 overexpression in mice results in cleft palate, while Fgf10, a loss of which also results in cleft palate, seems to function in cooperation with Wnt signaling (Alappat et al., 2005; Wu et al., 2015). Knockout of other Fgf genes (Fgf9 and Fgf18) and their receptors (Fgfr1 and Fgfr2) has also been associated with a cleft palate phenotype (Liu et al., 2002; Trokovic et al., 2003; Rice et al., 2004) (Fig. 2). Pax9 may feed back into and regulate canonical Wnt/β-catenin signaling in the anterior palatal mesenchyme during palatogenesis. Pax9 ablation causes an increase of the Wnt signaling modulators dickkopf1 Wnt signaling inhibitor 1 and 2 (Dkk1 and Dkk2), and intravenous delivery of small-molecule Dkk inhibitors can rescue the cleft palate phenotype in utero in Pax9-null mice.
| Gene   | Role                                    | Phenotype                                                                 | Interaction with Wnt signaling                                                                 | Orofacial mechanism                                                                                 | References                                                                                       |
|--------|-----------------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| ALDH1A3| Enzyme that produces RA from retinaldehyde | Mutations linked with craniofacial defects in both humans and mice; no oral clefts reported | Aldh1a3 represses the Wnt pathway by increasing RA levels, and is in turn repressed by canonical Wnt signaling | Required in the palatal epithelium to enhance RA signaling and downregulate target genes, possibly by repressing Wnt signaling | Dupé et al., 2003; Song et al., 2009; Kato et al., 2013; Osei-Sarfo and Gudas, 2014 |
| CYP26B1| RA-degrading enzyme                      | Cyp26b1-deficient mouse embryos have cleft palate                         | Loss of function results in increased RA signaling and reduced Wnt signaling                    | Modulates RA levels to repress Wnt signaling in palatal shelf mesenchymy                             | Okano et al., 2012; Hu et al., 2013 |
| FGF8   | FGF signaling ligand                    | Mesenchymal overexpression results in complete cleft palate in mouse       | Downstream of canonical Wnt signaling                                                          | Fgf signaling is regulated by Wnt signaling in facial ectoderm and anterior neural ridge            | Jin et al., 2011; Wang et al., 2011; Wu et al., 2015 |
| FGF10  | FGF signaling ligand                    | Fgf10-null mutant mice display open palate                                 | Antagonized by RA signaling and functions in cooperation with Wnt signaling                    | Fgf18 is an effector of Wnt signaling required for appropriate bone development                     | Liu et al., 2002; Reinhold and Naski, 2007; Wan et al., 2009; Koneczny et al., 2015 |
| FGF18  | FGF signaling ligand                    | Fgf18-deficient mice display highly penetrant cleft palate. Association found between SNPs and NSCLP in human populations | Canonical Wnt signaling directly activates Fgf18                                              | Conditional mutants show reduced Wnt1 expression, and less diffuse E-cadherin expression at the MEE | Dodé et al., 2003; Riley et al., 2007; Trokovic et al., 2003; Wang et al., 2013b |
| FGFR1  | FGF receptor                            | Cleft palate after conditional ablation or partial loss of Fgf1r1 in mice; linked to Kallmann syndrome and NSCLP in humans | Fgf signaling through Fgf1r1 is required for activation of Wnt signaling components             | Pitx2 may help orofacial cells to compensate for Fgf signaling deficiency                           | Slaney et al., 1996; Riley et al., 2007; Hosokawa et al., 2009 |
| FGFR2  | FGF receptor                            | Epithelial ablation results in cleft palate in mice; linked to Apert syndrome and NSCLP in humans | Fgf and Wnt proteins may interact through Pitx2                                               |                                                                                                    |                                                                                                  |
| TGFB2  | TGF signaling receptor                  | Cleft palate in mice after conditional ablation of Tgfbr2                 | Mutants show altered Wnt/tι3-catenin signaling due to Dkk dysregulation                         | Wnt signaling is required to establish Tgf signaling, which mediates secondary palate fusion      | Ito et al., 2003; Iwata et al., 2014 |
| TGFB3  | TGF signaling ligand                    | Association with NSCLP in Chilean families; the MEE fails to fuse in mice lacking Tgfβ3, causing fully penetrant cleft plate | Wnt signaling is required to establish Tgf signaling, which mediates secondary palate fusion      |                                                                                                    | Proetzel et al., 1995; Kaartinin et al., 1997; Suazo et al., 2010; He et al., 2011; Lane et al., 2014 |
| SHH    | Hedgehog signaling ligand               | In utero hedgehog signaling antagonism results in CLP; gain-of-function mutants display full CLP in mice | Shh is activated by β-catenin in the developing palate; it can also feed back to modulate Wnt signaling | Canonical Wnt/β-catenin signaling is required for appropriate Shh expression throughout palatal development | Huang et al., 2007; Lipinski et al., 2010; Lin et al., 2011 |
| GLI3   | Effector of hedgehog signaling          | SNPs associated with NSCLP in human patients; null mice exhibit cleft palate | The Gli3 repressor interacts with the activation domain of β-catenin and attenuates its activity; Shh signaling through Gli3 can be controlled by Wnt signaling | Cleft palate is thought to be due to abnormal tongue development in Gli3-null mice, rather than palatal shelf growth or fusion | Borycki et al., 2000; Ulloa et al., 2007; Huang et al., 2008; Wang et al., 2017b |
| KIF3A  | Primary ciliary intracellular transport protein | Knockout mice display cleft palate                                           | Inhibitory effects on Wnt signaling by cilia; also affects phosphorylation of Dvl proteins     | Primary cilia are required for midline cranial neural crest cells to respond to Wnt signals from surrounding epithelia | Liu et al., 2014b; Tian et al., 2017 |
| IFT88  | Intraflagellar transport complex component expressed in primary cilia | SNP in IFT88 identified in family with inherited CLP; neural crest-specific LOF in mice results in bilateral CLP | Ift88 represses canonical Wnt signaling and regulates Shh activity                             | Ciliary transport modulates Wnt activity, possibly through its role in Shh signaling in orofacial primordia | Corbit et al., 2008; Chang and Serra, 2013; Tian et al., 2017 |
| SFRP   | Secreted antagonists of WNT signaling   | Loss of Sfrp causes anterior palate extension failure and lower jaw agenesis in fish | Sfrp5 downregulates β-catenin activity, and expression is attenuated by the loss of Shh signaling in primary cilia |                                                                                                    |                                                                                                  |

Continued
Table 2. Continued

| Gene       | Role                                      | Phenotype                                                                 | Interaction with Wnt signaling                                                                 | Orofacial mechanism                                                                 | References                                        |
|------------|-------------------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------------------------|
| NOG        | Secreted protein that regulates growth factor gradients during embryonic patterning | Neural crest-ablation of noggins results in complete secondary cleft in mice | Modulates Bmp signaling downstream of Wnt pathway; shown to attenuate Wnt signaling in the dental epithelium | Noggin regulation of signaling gradients is required for appropriate skull base morphology to allow palatal shelf closure | He et al., 2010; Matsui and Klingensmith, 2014; Yuan et al., 2015 |
| BMP2       | BMP signaling ligand                      | Mutations associated with syndromic and non-syndromic CLP                | Wnt signaling directly activates Bmp2, which can, in turn, also modulate canonical Wnt signaling activity | Wnt signaling controls Bmp signaling initiated by Bmp2 and Bmp4 in craniofacial patterning | Zhang et al., 2009; Zhang et al., 2013; Saket et al., 2016 |
| BMP4       | BMP signaling ligand                      | Mutations is associated with NSCLP in humans                             | Wnt signaling controls Bmp signaling                                                           | Bmp4 acts downstream of canonical Wnt signaling in the developing palate              | Chen et al., 2012; Alexander et al., 2014          |
| BMP7       | BMP signaling ligand                      | BMP7 SNP linked to NSCLP in Han population; LOF results in murine cleft palate, although epithelial ablation alone is insufficient to confer cleft phenotype | Loss of Wnt signaling reduces Bmp7 expression during palatogenesis                               | Bmp7 may play roles in multiple tissues to regulate appropriate palatal development | Lin et al., 2011; Kouskoura et al., 2013; Yu et al., 2015 |
| BMPR1A     | Type I BMP receptor                       | Mesenchymal ablation results in submucosal cleft palate and downregulated epithelial Shh signaling; ectopic Bmp can rescue cleft palate and Shh expression in Mx1-null mice | Ectodermal Wnt impairment attenuates Bmp signaling                                             | Regulates Shh downstream of Wnt and Mx1 during palatal bone formation                 | Zhang et al., 2002; Baek et al., 2011; Zhu et al., 2016 |
| ACVR1      | Type I activin receptor, and type I BMP receptor family member | Constitutive epithelial activation results in cleft palate in mice        | Possibly activated by Wnt signaling                                                           | Temporal expression and loss thereof is required for appropriate epithelial proliferation and cell death in palatal epithelial cells | Noda et al., 2016                                |
| SUMO1      | Encodes a peptide that covalently binds to target proteins as a post-translational modification | Partially penetrant CLP in haploinsufficient mice; SUMO1 SNPs are linked to NSCLP in humans | SUMO1 modifies β-catenin to enhance canonical Wnt signaling                                     | SUMOylation of β-catenin is required for efficient transcription of Wnt targets during palatogenesis | Akuraya et al., 2006; Song et al., 2008; Huang et al., 2015 |
| CDH1       | E-cadherin is required for epithelial cell adhesion | CDH1 mutations are associated with NSCLP in humans                        | E-cadherin directly interacts with β-catenin and can modulate intracellular levels. Its transcription is also regulated by Lef1 independent of β-catenin | E-cadherin helps prevent ectopic Wnt signaling by sequestering excess cytosolic β-catenin, and by enhancing the activity of its destruction complex | Maher et al., 2009; Naresh et al., 2007; Marie and Hay, 2013; Iittiwut et al., 2016 |
| HAND2      | Transcription factor, involved in patterning of neural crest-derived tissues | Conditional ablation, hypomorphic expression, and enhancer deletions of Hand2 all result in cleft palate phenotypes in mouse | Activated by Wnt signaling during craniofacial skeletal development. Acts upstream of Shh signaling | Bmp and Wnt signaling mediate Hand2 expression in the palate epithelium | Yanagisawa et al., 2003; Morikawa et al., 2007; Xiong et al., 2009; Alexander et al., 2014 |
| p63 (TP63) | Epithelial transcription factor involved in proliferation and cell fate | p63 mutations cause cleft palate in both humans and mice                  | Directly activates Irf6, and is activated by Wnt signaling in orofacial development. May also feed back into the Wnt pathway | p63 activates Irf6 and promotes proliferation in the palatal shelf epithelium. Its temporally-regulated degradation is important for appropriate apoptosis, as well as adhesion required for fusion of the MEE | van Bokhoven and Brunner, 2002; Truong et al., 2006; Thomasen et al., 2008; Thomasen et al., 2010; Ferretti et al., 2011; Wu et al., 2012; Katoh et al., 2016 |
| IRF6       | Transcription factor                      | Mutations associated with NSCLP, linked to Van der Woude syndrome         | Activated by Wnt signaling via TCF/β-catenin                                                   | Effector of Wnt control of proliferation via p63                                       | Rahimov et al., 2008; Ferretti et al., 2011; Letra et al., 2012; Kurokawa et al., 2014 |
| MSX1       | Transcription factor, promotes outgrowth of maxillary prominences | Msx1-deficient mutants present with cleft palate. Linked to NSCP in human populations | Activated by canonical Wnt signaling through Lrp5β6, as well as directly downstream of Bmp pathway | Mediator of interaction between Wnt and Bmp pathways in palatogenesis                | Satokata and Maas, 1994; Song et al., 2000; Salahshourifar et al., 2011; Medio et al., 2012 |
| Gene | Role | Phenotype | Interaction with Wnt signaling | Orofacial mechanism | References |
|------|------|-----------|-------------------------------|---------------------|------------|
| **MSX2** | Transcription factor, important for craniofacial development | Compound mutants with Msd display severe facial defects, including cleft palate | Activated by canonical Wnt signaling through Lrp5/6 | Acts at the interface between Wnt and Bmp pathways | Ishii et al., 2003; Ishii et al., 2005; Song et al., 2009 |
| **OSR2** | Transcriptional repressor | Null mutant mice exhibit cleft palate | Regulates expression of Wnt signaling antagonists Dkk2 and Sfrp2 | Modulates several signaling pathways, including Bmp, Wnt and semaphorin, in palate and tooth development | Lan et al., 2004; Jia et al., 2016; Fu et al., 2017 |
| **GRHL3** | Epithelial transcription factor | Mutations linked to Van der Woude syndrome, as well as NSCLP and NSCPO; Grhl3-null mice have low penetrant cleft palate | Downstream effector of canonical Wnt5a-catenin signaling in epithelial tissues | Grhl3 is required for periderm differentiation and also plays a role in palate formation | Peyrard-Janvid et al., 2014; Kimura-Yoshida et al., 2015; Wang et al., 2016; Eshele et al., 2018 |
| **SHOX2** | Transcription factor expressed in the anterior secondary palate | Shox2 expression is altered by canonical Wnt signaling in mouse palatogenesis | Proliferation and palate closure is differentially regulated along the A-P axis | | Yu et al., 2005a; Lin et al., 2011 |
| **TFAP2A** | Transcription factor in neural crest lineages | Mutations cause fully penetrant cleft palate in mice. Haploype analyses linked TFAP2A with familial NSCLP | Wnt signaling activates Tfp2a expression during neural crest induction | Tfp2a may link Wnt signaling to Fgf signaling and expression of proliferation via p63 and Irf6 | Martinelli et al., 2011b; Green et al., 2015; Leung et al., 2016 |
| **GBX2** | Transcription factor involved in neural crest induction | Gbx2 mutant mice display partially penetrant CPO | Direct target of canonical Wnt signaling | Effector of Wnt signaling that establishes cell specification boundaries | Byrd and Myers, 2005; Li et al., 2009 |
| **PAX7** | Transcription factor required for neural crest development | Intergenic SNPs near PAX7 linked to NSCLP in human GWAS | Pax7 was shown to repress Wnt signaling in vitro | Pax7 is a repressor of Wnt signaling and acts antagonistically to Barx2 in regulating Wnt signaling | Zhuang et al., 2014; Leslie et al., 2015 |
| **PAX9** | Transcription factor required for secondary palate development | Mutations linked to NSCLP susceptibility in humans. Fully penetrant cleft palate in mice | Downstream effector of Wnt signaling in the palate mesenchyme, upstream modulator of Dkk expression | Imporotant orofacial regulator, may act as interface between Wnt, Fgf and Bmp signaling pathways | Neubüser et al., 1997; Mensah et al., 2004; Ichikawa et al., 2006; Jia et al., 2017a; Li et al., 2017 |
| **RUNX2** | Transcription factor important for osteoblast differentiation | Mutations linked to cleidocranial dysplasia, which can include CLP, both in human patients and mouse models | Direct transcriptional target of Wnt5a-catenin signaling. Runx2 was shown to enhance Wnt signaling by repressing Axin2 expression | Runx2 is an important effector of Wnt signaling in craniofacial morphogenesis | Otto et al., 1997; Cooper et al., 2001; Gaur et al., 2005; McGee-Lawrence et al., 2013 |
| **VAX1** | Ectodermal transcription factor | Mouse mutants display cleft palate. VAX1 locus associated with NSCLP in GWAS | Vax genes antagonize Wnt signaling | Cleft palate appears to be indirect due to effects of cranial dysmorphogenesis | Hallonet et al., 1999; Mangold et al., 2010; Vacik et al., 2011; Geoghegan et al., 2017 Schiessinger et al., 2009; Leslie et al., 2012 |
| **ARHGAP29** | GTPase activator involved in cytoskeletal regulation | Association found between genomic variants and NSCLP | The Rho pathway acts downstream of non-canonical and canonical Wnt signaling. Arhgap29 mutations show altered Irf6 expression | Irf6 regulatory network likely interacts with the Rho pathway through Arhgap29 | |
| **FOXF2** | Forkhead-box transcription factor, regulated by Shh | Conditional ablation results in CLP in mice while cultured knockout palate explants fail to fuse. SNPs linked to human NSCLP | Foxf2 acts downstream of Shh in oral cavity tissues, and can also enhance and repress mesenchymal Tgfβ and Wnt signaling, respectively | Suppresses Wnt signaling to regulate mesenchymal proliferation | Ormestad et al., 2006; Bu et al., 2015; Nik et al., 2016; Xu et al., 2016; Eversen et al., 2017 |
| **TBX1** | T-box transcription factor | Loss of function in mice leads to cleft palate | Downregulated by RA, activated by Wnt signaling | Wnt repression by RA also represses the Wnt target gene Tbx1 | Okano et al., 2008; Funato et al., 2012 |
| **TBX22** | T-box transcription factor | Loss of function in mice leads to cleft palate. Mutations linked to human cleft palate patients | Tbx22 is repressed by Bmp signaling and activated by Fgf signaling | Transcriptional repressor regulated by several signaling pathways during palatogenesis | Marciano et al., 2004; Pauws et al., 2009; Fuchs et al., 2010 |
Homeobox transcription factor involved in tissue renewal

| Gene | Role | Interaction with Wnt signaling | Orofacial mechanism | References |
|------|------|-------------------------------|--------------------|------------|
| **PITX2** | Transcription factor important for pituitary, heart and brain development | Loss of function results in cleft palate in mouse | Pitx2 is activated by canonical Wnt signaling, and can also regulate expression of several Wnt ligands | Kiousi et al., 2002; Liu et al., 2003; Iwata et al., 2012; Basu and Roy, 2013 |
| **HOXA2** | Transcription factor important for craniofacial development | Hoxa2-null mice display cleft palate, and loss of function slows palatal shelf fusion in culture | Hoxa2 modulates Bmp signaling and directly regulates transcription of several Wnt pathway components, including Wnt5α and Fzd4 | Smith, et al., 2009; Donaldson et al., 2012; Iyvanar and Nazarali 2017 |
| **SIX2** | Homeobox transcription factor | Palatal shelves fail to extend to the midline in Six2-null mouse embryos | Six2 represses signaling through β-catenin and acts downstream of Hoxa2 | Self et al., 2006; Park et al., 2012; Okello et al., 2017; Sweat et al., 2018 |

**Wnt-Bmp-Shh signaling crosstalk**

The homeobox-containing Msx transcription factors function as downstream effectors of Bmp signaling in many developmental processes, including in palateogenesis (Cheng et al., 2003; Tribulo et al., 2003; Hayashi et al., 2006; Parada and Chai, 2012). Bmp signaling directly activates Mxs genes at early stages of ectodermal patterning in order to specify the neural crest (Graham et al., 1994; Tribulo et al., 2003). Lrp6-mediated Wnt signaling also regulates Mxs1 and Mxs2 expression in the orofacial primordia, but Lrp6 ablation does not affect Bmp4 during primary lip/palate formation and fusion, suggesting that the Bmp and Wnt pathways may converge by way of common activation of Mxs1/Mxs2 (Song et al., 2009) (Fig. 2). Mice with knockouts of either Mxs1 or the Bmp receptor Bmpr1a display cleft palate and downregulated Shh signaling. Bmp4 expression in the anterior palate mesenchyme is lost in Mxs1-null mice, while transgenic ectopically expressed human BMP4 is able to rescue both Shh activity and the cleft palate phenotype in these animals (Zhang et al., 2002; Back et al., 2011). This implies that Bmp proteins are the primary effectors of Mxs1 activity, and that an Msx1-Bmp-Shh cascade may act downstream of Lrp6-mediated Wnt signaling to regulate palateogenesis. This link between Bmp and Shh signaling in the palate primordia appears to be mediated by the epithelial transcription factor heart and neural crest derivatives expressed 2 (Hand2) (Xiong et al., 2009).

Many Bmp receptors are expressed in differing patterns along the anterior-posterior (A-P) axis of the palatal shelves during palateogenesis. Submucous cleft palate (Box 1) results from the overexpression of the Bmp receptor activin A receptor-type I (Acvr1) (Noda et al., 2016), so appropriate levels and localization of Bmp pathway activity appear critical for correct tissue responses to palatogenic signaling. Conditional deletion of the Bmp signaling receptors Bmpr1a or Acvr1 in neural crest cells results in multiple craniofacial abnormalities, including submucous cleft palate (Dudas et al., 2004; Saito et al., 2012) (Fig. 2). Interestingly, Wnt9b may regulate Bmp4 during lip fusion (Lan et al., 2006), but this interaction has not been demonstrated during palate fusion. Wnt5a, however, is expressed in a descending gradient from the anterior to the posterior developing palate, and it can act as a negative regulator of Bmp4 in a concentration-dependent manner across the palate (He et al., 2008). Bmp2 seems unaffected by Wnt5a, thus Bmp2 activation may occur in a separate pathway from that of Bmp4 (He et al., 2008). Bmp7 is also expressed in the developing palate and during rugae (Box 1) formation, where it acts downstream of canonical Wnt signaling (Lin et al., 2011), and has been linked with cleft palate in both humans and mice (Kouskoura et al., 2013; Yu et al., 2015). Bmp signaling is upregulated in homeobox A2 (Hoxa2)-null embryos, and Hoxa2 may inhibit palatal osteogenic differentiation from mesenchymal cells via its modulation of Bmp signaling (Iyvanar and Nazarali, 2017). In addition, homeobox protein sine oculis-related homeobox 2 (Six2) likely acts as a downstream effector of Hoxa2 in regulating mesenchymal cell proliferation during secondary palate formation (Okello et al., 2017), and palatal shelves fail to extend to the midline in Six2 knockout mice (Sweat et al., 2018). It remains unclear whether this activity is related to the interaction of Hoxa2 with Bmp. Six2 is known to repress Wnt/β-catenin by binding to T-cell factor/lymphoid enhancer binding factor 1 (Tcf/Lef1) family members during nephrogenesis (Self et al., 2006; Park et al., 2012), but it remains unclear whether Six2 does so during palateogenesis.
Wnt-Tgfb signaling crosstalk

Epithelial Wnt/β-catenin signaling also regulates Tgfb signaling. Wnt-mediated Tgfb3 activation is required for MEE cell apoptosis during palate shelf closure (He et al., 2011) (Fig. 2), and knockout of all three isoforms of Tgfb has been associated with cleft palate in mice, in either single or doubly mutant lines (Kaartinen et al., 1995; Sanford et al., 1997; Jin and Ding, 2014) (Fig. 2). Tgfb1 and Tgfb3 are semi-redundant, and overexpression of Tgfb1 can partially rescue the cleft phenotype observed in Tgfb3-null mice (Yang and Kaartinen, 2007). Mutations in transforming growth factor beta receptor 3 (Tgfb3, also known as betaglycan), which binds Tgfb ligands without transducing the signal, cause cleft palate due to reduced cell proliferation and increased apoptosis (Hill et al., 2015) (Fig. 2). By contrast, conditional Tgfb1 and Tgfb2 knockout in neural crest cells also causes cleft palate and skull defects due to insufficient cell proliferation (Ito et al., 2003; Dudas et al., 2006) (Table 2, Fig. 2). Tgfb signaling through epithelial Tgfb2 feeds back into the Wnt pathway by repressing Dkk1 and Dkk4 to enhance mesenchymal Wnt signaling activity (Iwata et al., 2014). The forkhead box transcription factor Foxf2, which represses Wnt signaling in the gastrointestinal system (Ormestad et al., 2006), may effect Tgfb signaling during palate development, and has been linked to orofacial clefts in both mice and humans (Bu et al., 2015; Nik et al., 2016) (Table 2, Fig. 3). Foxf2 ablation downregulates Tgfb2 during palatogenesis, causing a decrease in mesenchymal cell proliferation and aberrant collagen accumulation (Nik et al., 2016), resulting in cleft palate in mice (Wang et al., 2003) (Table 2, Fig. 3). Repression of Wnt signaling by Foxf2 has been demonstrated in intestinal fibroblasts (Nik et al., 2013), although a direct relationship between Foxf2 and Wnt signaling during palatogenesis remains undemonstrated.

Wnt-Shh-cilia crosstalk

Hedgehog signaling during embryogenesis depends on primary cilia function and intraflagellar transport (Huangfu et al., 2003; Huangfu and Anderson, 2005) (Fig. 3). Individuals with ciliopathies resulting from defects of the primary cilia often have CLP, and tissue-specific deletion of the intraflagellar transport genes intraflagellar transport 88 (Ift88) or kinesin family member 3A (Kif3a) in mice causes CLP (Liu et al., 2014b; Schock et al., 2017; Tian et al., 2017) (Fig. 3). Mouse pups with conditional deletion of Ift88 in cranial neural crest cells with Wnt1-driven Cre die at birth due to severe craniofacial defects, including bilateral CLP (Box 1), whereas elimination of Ift88 specifically in the palatal mesenchyme results in CPO (Tian et al., 2017). Loss of Ift88 results in a downregulation of Shh signaling in the palatal mesenchyme (Tian et al., 2017). In addition, a novel missense mutation in IFT88 has been reported in a family affected by isolated CLP, suggesting it as a candidate gene for orofacial clefts (Tian et al., 2017). Both Ift88 and Kif3a may repress canonical Wnt signaling (Corbit et al., 2008; Chang and Serra, 2013) (Fig. 3). Combined, these results underscore the significance of intraflagellar proteins in craniofacial development, which involves Shh signaling, and the role of Shh signaling in feeding back to negatively regulate Wnt signaling.

The ventral anterior homeobox (Vax) transcription factors are important for neural patterning, and they mediate signaling between Shh and Wnt (Vacic et al., 2011). Sfrp and ventral anterior homeobox 1 (Vax1) are the downstream effectors of Shh signaling, and Shh, in turn, inhibits Wnt/β-catenin signaling (Kurosoaka et al., 2014) (Fig. 3). Furthermore, VAX1 is a candidate human NSCLP gene (Mangold et al., 2010), and Vax1-null mouse embryos exhibit cell proliferation problems during cranial development around E10.5, possibly due to a downregulation of Shh. These embryos do not present with a CLP phenotype, suggesting that Vax1 does not play a direct role in palatogenesis (Geoghegan et al., 2017). Palatal rugae are established by Shh expression, which is opposed by Fgf signaling at the interrugal regions of the epithelium (Economou et al., 2012). Wnt/β-catenin signaling is also required for Shh induction in the palatal rugae (Lin et al., 2011; Kawasaki et al., 2018).

Wnt5a/Rox2 may act upstream of the non-canonical Wnt signaling molecule Prickle1 (Liu et al., 2014a; Yang et al., 2014) (Fig. 1), and Prickle1 itself may act upstream of Shh during palatogenesis (Yang et al., 2014) (Table 2, Fig. 3). In osteoblast-lineage cells, the non-canonical and canonical Wnt pathways have a positive relationship, and Wnt5a upregulates Wnt/β-catenin signaling, while its ablation inhibits canonical signaling by reducing Lrp5/Lrp6 expression (Okamoto et al., 2014). Wnt5a may also act upstream of Msx1, Bmp2, Bmp4 and Shh during palatogenesis, placing Wnt5a as a promising candidate for signaling pathway crosstalk during palate development (He et al., 2008; Smith et al., 2012). Although Msx1 expression was downregulated in Wnt5a knockout mouse palates, an Msx1-binding enhancer was identified upstream of Wnt5a, implying a possible synergistic relationship between these two factors (He et al., 2008; Nishihara et al., 2016).

Shh has been reported to activate Fox genes during lip and palate development (Jeong et al., 2004; Nik et al., 2016; Everson et al., 2017). Foxf2 also represses Fgf18 signaling from the palatal mesenchyme, which itself negatively regulates Shh expression in the palatal epithelium, leading to reduced Shh expression in Foxf2 knockout mice (Xu et al., 2016), which suggests a positive-feedback loop (Fig. 3). Gli3 acts as an activator of hedgehog pathway targets in the presence of Shh signaling and becomes a repressor when Shh signaling is absent (Wang et al., 2000). Moreover, Gli3 has been associated with NSCLP in human patients (Wang et al., 2017b), and...
Gli3-null mouse embryos exhibit cleft palate and tongue abnormalities due to improper tongue morphogenesis and failure of palatal shelf elevation and fusion (Table 2, Fig. 3) (Huang et al., 2008). The repressor form of Gli3 modulates Wnt signaling and physically interacts with β-catenin, linking the Shh and Wnt pathways (Ulloa et al., 2007).

**Wnt-RA-Fgf signaling crosstalk**

RA plays an important role in normal palatogenesis (Okano et al., 2014), and excess RA exposure in human and murine embryos can cause orofacial clefts (Abbott and Pratt, 1987; Abbott et al., 1989). Several aldehyde dehydrogenases are involved in the synthesis of RA from retinaldehyde, with aldehyde dehydrogenase family 1, subfamily A3 (Aldh1a3) being largely responsible for RA production in the oral epithelium (Kato et al., 2013). RA signaling interacts with the Wnt/β-catenin pathway (Fig. 4) (Kumar and Duester, 2010; Yasuhara et al., 2010; von Gise et al., 2011; Zhao and Duester, 2009; Osei-Sarfo and Gudas, 2014), and alters cellular proliferation and apoptosis in the craniofacial mesenchyme and epithelium through its repression of Wnt signaling in palatogenesis (Hu et al., 2013). Canonical Wnt signaling appears to feed back into and inhibit RA signaling, as Aldh1a3 is ectopically expressed in the upper lip primordia of Lrp6-deficient embryos (Song et al., 2009) (Fig. 4). Cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1), the enzyme that degrades RA and therefore regulates endogenous RA levels, is required for proper elevation of palatal shelves, and Cyp26b1 knockout mice display cleft palate due to excess RA (Okano et al., 2012). Cyp26b1 enhances T-box 1 (Tbx1) and Fgf10 expression in the oral epithelium, while an excess of RA represses both. Fgf10 expression is lost in Cyp26b1-null mice, and palatal Tbx1 expression was downregulated when murine fetuses were treated with exogenous RA (Okano et al., 2008; Okano et al., 2012) (Fig. 4). Both Tbx1-null and Fgf10-null mice display cleft palate (Alappat et al., 2005; Funato et al., 2012), suggesting that these important regulators of palatal shelf elevation act downstream of Cyp26b1, and their expression is likely modulated by RA levels. As researchers continue to identify the factors that connect these different pathways, it becomes increasingly important to understand how they are regulated.

**Crosstalk of Wnt signaling with epigenetic regulators in orofacial clefts**

Sequence-independent gene regulatory mechanisms, such as histone modification, DNA methylation and microRNA (miRNA) transcript regulation, have garnered increasing attention in recent years. These epigenetic mechanisms play a role in regulating many Wnt pathway components (Wils and Bijlsma, 2018). Studies suggest that miRNAs are involved in regulating Wnt signaling during palatogenesis; in mice, conditional deletion of Dicer1, the key effector of RNA interference (RNAi)-mediated mRNA cleavage, leads to craniofacial defects, including cleft palate (Zehir et al., 2010). A 2016 study of plasma miRNAs expressed in human NSCLP patients suggests that many key targets of dysregulated miRNAs share functional relationships with Wnt, Notch, hedgehog and lipid signaling pathways (Li et al., 2016). The miRNAs hsa-miR-24-3p, hsa-miR-1260b and hsa-miR-205-5p have been identified in a human transcriptome screen as candidates for NSCLP, and were computationally predicted to target several Wnt signaling pathway components (Wang et al., 2017a). Another miRNA, miR-544a, has been associated with downregulation of CDH1 during EMT in cancer cells, in turn activating the Wnt signaling pathway (Yanaka et al., 2015). The miR-17-92 cluster reportedly targets transcripts of NSCLP-associated Wnt target genes Tbx1 and Tbx3, and is itself a target of Bmp signaling and of the craniofacial pioneer factor AP-2α. miR-17-92 knockout in mouse embryos results in severe craniofacial defects, including CLP, the severity and penetrance of which are increased in miR-17-92,miR-106b-25 compound mutants (Wang et al., 2013a). In zebrafish, platelet-derived growth factor (Pdgf) signaling is an important regulator of palatogenesis, and it is modulated by miR-140 during palatogenesis (Eberhart et al., 2008). Further studies discerning whether the described roles of miRNAs in other cellular processes resemble their roles in palatogenesis may contribute to our understanding of the mechanisms by which cleft palate arises.

Several studies examined epigenetic modifications in NSCLP, including DNA methylation (Alvizi et al., 2017; Sharp et al., 2017). The transcription factor and human CLP candidate SOX4, the targets of which include FZD5 (Scharer et al., 2009), has been implicated in studies of genomic regions that are differentially methylated during palatogenesis (Seelan et al., 2012). Changes in CDH1 promoter methylation levels in human blood and lip tissue have been correlated with NSCLP, as well as with differences in NSCLP penetrance in susceptible families, implying that DNA methylation patterns may account for the variable penetrance of CLP phenotypes (Alvizi et al., 2017). A stochastic deficiency in DNA methylation of a retrotransposon near the coding region of Wnt9b in the A/WySn mouse impaired its transcription and contributed to an incompletely penetrant CLP phenotype (Juriloff et al., 2014). The role of histone modifications in craniofacial development is less understood, but recent studies suggest that...
histone H3 acetylation can play a role in the formation of cleft palate in mouse due to dysregulation of Tgfb signaling, although how this process affects Wnt signaling has not been demonstrated (Yuan et al., 2016). However, the histone acetyltransferase p300 (also known as Ep300) is important for gene regulation, and its ablation in mouse palatal mesenchyme cells results in altered Wnt signaling, as well as in aberrant Wnt-dependent proliferation and migration (Warner et al., 2016). An improved understanding of epigenetic regulation of Wnt signaling and related pathways may hold the key to addressing the impact of environmental and non-genetic factors on the presentation of orofacial clefts.

Translational perspectives
To date, the most prevalent treatment for orofacial clefts is surgical repair coupled with nasoalveolar molding to direct postnatal tissue growth and subsequent orthodontic treatment (Chen et al., 2005). Protocols and procedures have varied widely, not only in developing areas of the world, but within developed countries as well (Moosley et al., 2009). A better understanding of the complex interactions between components of the Wnt and other signaling pathways that govern lip/palate formation will provide better opportunities for treatment and prevention of orofacial clefts through cellular- and molecular-based methods, reducing the need for surgical intervention (Panetta et al., 2008). Research in animal models has identified several altered pathways that, when targeted, reversed orofacial clefts. Direct modulation of Wnt signaling by chemically stabilizing a catalytically inactive allele of the canonical Wnt pathway factor Gsk3 has shown therapeutic potential in mice, where its timely reactivation could reverse a cleft palate phenotype in Gsk3β-deficient mice (Liu et al., 2007). Additionally, ectopic expression of Wnt in the ectoderm rescued the orofacial cleft phenotype in Pbx-deficient mouse models (Feretti et al., 2011). Modulation of Shh signaling has also been shown to rescue cleft palate in the Msx1-null mouse model, both through ectopic Bmp expression and through downregulation of distal-less homeobox 5 (Dlx5) (Zhang et al., 2002; Han et al., 2009). Reynolds and colleagues have shown that administration of either 3-4 mg/kg folic acid or 140-187 mg/kg methionine to pregnant mice that were previously treated with intraperitoneal RA to induce CLP reduces the frequency of cleft palate to 6%, compared with 76% in RA-treated controls. Interestingly, the combined folic acid and methionine treatment completely rescued the RA-induced aberrant palatogenesis (Reynolds et al., 2003).

Utilizing controlled intravenous delivery of the small-molecule Wnt agonists WAY-262611 and IIIc3a (both acting as Dkk inhibitors) into Pax9 mutant mice rescues the growth and fusion of palatal shelves by restoring Wnt signaling (Jia et al., 2017a; Li et al., 2017). In addition to small-molecule modulation, synthetic ligand analogs have also shown potential to stimulate Wnt signaling (Andersson et al., 2015; Zhan et al., 2017) and could lead to the development of future treatments for orofacial clefts. Additionally, genetic inactivation of Wise (also known as Sostdc1), a canonical Wnt antagonist, in Pax9-deficient mouse embryos rescued the palatal shelf elevation, mainly through restoring hyaluronic acid accumulation in the palatal mesenchyme (Li et al., 2017). Wnt5a analogs, such as Foxy-5 or Box-5, currently used in cancer research (Andersson et al., 2015; Zhan et al., 2017), may also serve as a treatment approach for orofacial clefts by targeting non-canonical Wnt signaling, warranting future research. Taken together, these reports indicate that Wnt signaling modulators could contribute to an effective molecular treatment regime for orofacial clefts.

Given the heterogeneous causes of orofacial clefts and the variability in the genotypes of affected individuals, it is unlikely that we will see a ‘one-size’ approach to non-surgical orofacial cleft treatment any time soon. However, successful studies using mutant mouse models are promising for the prospect of pathway-specific treatments to allow for prenatal intervention when a fetal genotype renders human embryos at risk of orofacial cleft development. Implementation of promising therapies to human patients would impinge on the timing of application and on the accurate detection of improper palatogenesis. Even if parents carrying alleles linked to orofacial clefts were to commit to prenatal genotyping, many palatal development processes occur early in gestation. Even with improved protocols for correcting the levels of a target signaling factor in patients, implementing postconception measures at such an early stage of pregnancy, before many mothers know that they are pregnant, is challenging. Further challenges arise from the high variability in the manifestation and penetrance of orofacial cleft phenotypes. The complexity of the molecular processes that govern orofacial development makes it difficult to predict the therapeutic requirement. Moreover, a particular intervention may not be applicable to more than a minor subset of cases, even in individuals with defects in a particular gene or pathway. A deeper understanding of the pathways that govern palatogenesis may allow in vitro fertilization with selected gametes that do not possess the risk-imparting allele.

Interactions between Wnt/β-catenin signaling and other morphogenetic signaling pathways are widely employed in many different developmental programs. The nature of embryonic development creates significant potential for off-target effects and disruption of other essential developmental processes in both mother and child if molecular treatments to correct palatogenesis errors are applied systemically. Before treatments targeting signaling pathways could be considered for clinical trials with human patients, such risks would need to be thoroughly explored and addressed, and likely require new delivery techniques more advanced than those currently available. Additionally, because NSCLP is not life threatening, many parents may be unwilling to attempt untested and potentially dangerous approaches, despite the burden and difficulty of current treatments. However, in the future, a more complete understanding of morphogenetic pathway crosstalk and the systemic impact of perturbations to them may eventually allow the progression of molecular clinical approaches to a point at which they are considered safe. A robust understanding of how signaling pathways function in all systems and processes during development will be able to not only inform studies related to orofacial clefts, but also contribute to the development of treatments for other syndromes and disorders with Wnt pathway etiologies. Despite the many barriers that still remain, knowledge of developmental mechanisms is helping to, and will continue to, facilitate the refinement of techniques for the application of that knowledge to develop the means to safely and effectively treat congenital disorders like orofacial clefts.

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Author contributions
K.R., P.K., L.S.R., and R.G. collected and analyzed the references and wrote the manuscript; Y.J. and S.K. assisted with manuscript preparation; C.J.Z. conceptualized, advised, edited and approved the manuscript.
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