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Molecular Mechanisms of Midfacial Developmental Defects

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Abstract

The morphogenesis of midfacial processes requires the coordination of a variety of cellular functions of both mesenchymal and epithelial cells to develop complex structures. Any failure or delay in midfacial development as well as any abnormal fusion of the medial and lateral nasal and maxillary prominences will result in developmental defects in the midface with a varying degree of severity, including cleft, hypoplasia, and midline expansion. In spite of the advances in human genome sequencing technology, the causes of nearly 70 percent of all birth defects, which include midfacial development defects, remain unknown. Recent studies in animal models have highlighted the importance of specific signaling cascades and genetic-environmental interactions in the development of the midfacial region. This review will summarize the current understanding of the morphogenetic processes and molecular mechanisms underlying midfacial birth defects based on mouse models with midfacial developmental abnormalities.

Keywords

Midfacial development; neural crest cells; midfacial cleft; cell signaling; growth factor

Introduction

During embryogenesis, the midfacial region, defined as the nose, upper lip, maxilla, primary palate, and zygomatic bones, is derived from seven processes: the frontonasal process, the paired lateral and medial nasal processes, and the paired maxillary processes (Cox, 2004). This region is largely populated by mesenchymal cells derived from cranial neural crest (CNC) cells, which are covered with ectoderm (Helms et al., 2005). Interactions between the CNC cells and epithelial cells are crucial for normal midfacial development, but the cause of many defects remain unknown. Significant progress has been made in identifying both genetic and environmental risk factors for midfacial malformations such as midfacial cleft, hypoplasia, and hypertelorism (aka midline expansion). In this review, we will summarize the current understanding of the molecular mechanisms underlying midfacial birth defects.

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Midfacial Development and Birth Defects

In humans and mice, early development of the midfacial region depends upon the growth and fusion of several processes, including the frontonasal process (aka frontonasal prominence), the paired lateral and medial nasal processes, and the paired maxillary processes. In the fifth week of gestation in humans and at embryonic day 10 (E10) in mice, the surface ectoderm of the frontonasal process thickens and develops into two bilateral nasal placodes. The lateral and medial nasal processes emerge from the nasal placodes, with the nasal pits forming in the center. In humans, the maxillary process of the first branchial arch (aka first pharyngeal arch) grows medially and approaches the lateral and medial nasal processes to form the upper lip and push the medial nasal process toward the midline. The merging of the two medial nasal processes also results in the formation of the primary palate carrying the incisors. In mice, the fusion between the medial and lateral nasal processes begins at E11. Subsequently, the maxillary processes grow toward each other and establish contact, fusing in the midline at E13 and forming one continuous structure. Prior to fusion, the epithelium on the opposing primordium is intact, with only occasional disruptions in the basal lamina, whereas during fusion, contact and adhesion, together with physical forces, trigger the degeneration of the basal lamina and change the fate of epithelial cells (induction of programmed cell death and activation of cell migration of medial edge epithelial cells) (Jiang et al., 2006; Shuler, 1995). At the seventh week in humans and E13.5 in mice, the fusion of the midfacial region is completed through the disappearance of the epithelial seams between the processes (Fig. 1). Failure of any of the steps of midfacial morphogenesis (defects in growth and patterning of the mesenchyme or epithelial fusion) results in midfacial cleft, hypoplasia, and/or hypertelorism in humans and mice.

CNC cells are multipotent, proliferative cells originating from the dorsal aspect of the neural tube (Chai and Maxson, 2006). Facial processes arise during early embryogenesis from ventrally migrating CNC cells, in combination with ectoderm and mesoderm, and undergo directed growth and expansion around the nasal pits to fuse with one another (Jiang et al., 2006). CNC cells from the diencephalon and anterior mesencephalon give rise to the lateral and medial nasal processes, while those originating from the posterior mesencephalon and hindbrain give rise to the maxilla and mandible (Serbedzija et al., 1992). In addition, CNC cells acquire their anterior-posterior identities before migration. However, the patterning of CNC cells is determined by niches (i.e., reciprocal signals from the microenvironment), mainly at the post-migratory stage (Trainor et al., 2003). These findings suggest that each process may be regulated through temporal and spatial specific mechanisms to form each unique structure, and that CNC cells play a key role in the formation of the midfacial region.

Molecular Mechanisms of Midfacial Developmental Defects

The identification of causal genes of midface anomalies in humans will help us understand their underlying molecular mechanisms. Mutations in genes involved in major signaling pathways (FGF, WNT, TGFβ, BMP, PDGF, and Notch signaling) and microRNAs (miRs) have been postulated as contributing genetic factors in midfacial birth defects in addition to environmental risk factors (Fig. 2 and Table 1).

1) FGF Signaling Pathway

Fibroblast growth factors (FGFs) regulate mesoderm establishment, neural crest (NC) patterning, myogenesis, and morphogenesis during embryogenesis (Nie et al., 2006a). FGF signaling activation is regulated by their receptors (five distinct membrane FGF receptors) and ligands (at least 22 known different FGF ligands) (Powers et al., 2000). Mutations in FGF signaling molecules result in craniofacial malformations, which include midfacial anomalies.

Upstream of FGF Signaling—The function of the zinc finger transcription factor SP8 in FGF signaling is essential for proper bone formation. Mice with a deficiency of *Sp8* (*Sp8*$^{-/-}$ mice) exhibit severe midfacial defects via reduction of *Fgf8* and *Fgf17* expression (Kasberg et al., 2013). *Sp8*$^{F/F}$*;Pax3-Cre* (*Pax3-Cre* is expressed in NC cells and in early metanephric mesenchymal lineages) and *Sp8*$^{F/F}$*;Foxg1-Cre* (*Foxg1-Cre* is expressed in cells derived from the telencephalon) mice display midfacial clefts, exencephaly, and absence of frontal and parietal bones, as seen in *Sp8*$^{-/-}$ mice (Kasberg et al., 2013).

FGF Receptors—In humans, gain-of-function mutations in FGF receptors have been found in a series of midfacial hypoplasia and craniosynostosis (a premature suture closure) syndromes. Syndromes characteristic of midfacial malformations (e.g., hypertelorism and midfacial hypoplasia) and craniosynostosis include the Jackson–Weiss (OMIM 123150), Pfeiffer (OMIM 101600), Apert (OMIM 101200), and Crouzon syndromes (OMIM 123500) (Johnson and Wilkie, 2011; Kress et al., 2000; Roscioli et al., 2000; Senarath-Yapa et al., 2012). Pfeiffer syndrome is an autosomal dominant disorder caused by a gain-of-function mutation in the FGF receptor type 1 (*FGFR1*) gene [C>G transversion at position 755 of *FGFR1*, resulting in a proline to arginine substitution at amino acid 252 (*FGFR1*^{P252R})], whereas Jackson–Weiss syndrome, also an autosomal dominant disorder, is caused by a mutation in the FGF receptor type 2 (*FGFR2*) gene [C>G transversion at position 1043 of *FGFR2*, resulting in an alanine to glycine substitution at amino acid 344 (*FGFR2*^{A344G})]. Apert syndrome is also the result of gain-of-function mutations of *FGFR2*, over 99% of which are a serine to tryptophan substitution at amino acid 252 (*FGFR2*^{S252W}) or a proline to arginine substitution at amino acid 253 (*FGFR2*^{P253R}). In these syndromes, midfacial hypoplasia is often associated with a short cranial base, reduced pharyngeal height, narrow nasal cavity, and a diminished nasopharyngeal space leading to severe upper airway obstruction. In addition, other gain-of-function mutations in *FGFR2* and *FGFR3* have been identified in patients with Crouzon syndrome—an autosomal dominant condition characterized by midfacial hypoplasia, craniosynostosis, and ocular proptosis—and achondroplasia with midfacial hypoplasia [e.g., *FGFR2*^{C278F}, a cysteine to phenylalanine substitution at amino acid 278, and *FGFR2*^{C342Y}, a cysteine to tyrosine substitution at amino acid 342) (Nolan et al., 2013). Thus, particular mutations in FGF receptors seem to be strongly associated with the clinical features of these syndromes.

Fgfr1 and *Fgfr2* are widely expressed in the facial mesenchyme and ectoderm, respectively, both in mice and humans (Bachler and Neubuser, 2001; Britto et al., 2001; Wilke et al., 1997). Ablation of *Fgfr1* in CNC cells (*Fgfr1*$^{F/F}$*;Wnt1-Cre* mice) results in cleft lip, due to a CNC migration defect in the frontonasal process, and cleft palate, which is caused by a

proliferation defect in the palate (Wang et al., 2013a). Mice with a mesodermal deficiency of *Fgfr2* (*Fgfr2*$^{F/F}$*;Dermo1-Cre* mice) exhibit midfacial hypoplasia and domed-shaped skulls (Yu et al., 2003). By contrast, *Fgfr2*$^{F/F}$*;Wnt1-Cre* and *Fgfr3*$^{-/-}$ mice show no midfacial defects (Pan et al., 2008; Valverde-Franco et al., 2004). These findings suggest that individual FGF receptors have different distribution and involvement in murine midfacial development. Gain-of-function mutations in *FGFR2* in mice (*Fgfr2*$^{+/P253R}$, *Fgfr2*$^{+/S252W}$, *Fgfr2*$^{+/S252W}$*;Mesp1-Cre*, and *Fgfr2*$^{+/S252W}$*;Wnt1-Cre* mice) result in skull malformations including midfacial hypoplasia, as seen in patients with these mutations (Holmes and Basilico, 2012; Wang et al., 2010; Yu et al., 2000).

FGF Ligands—Mutations in the *FGF8* gene have been found in patients with Kallmann-like idiopathic hypogonadotropic hypogonadism, midfacial hypoplasia, and cleft lip and palate (Stanier and Pauws, 2012). *FGF8* mutations are also found in some cases of Crouzon syndrome (Li et al., 2013).

In mice, *Fgf8* is broadly expressed in the midfacial ectoderm at E9.5; however, at later developmental stages, namely at E10.5 and E11.5, the expression is spatially restricted to the edge of the nasal pit and the oral edge of the medial nasal process (Bachler and Neubuser, 2001). Corresponding to the *Fgf8* expression pattern, mice with inactivation of *Fgf8* in the first branchial arch ectoderm (*Fgf8*$^{F/F}$*;Nes-Cre* mice: *Nes-Cre* is expressed in the maxillary process mesenchyme, oral and dental epithelium, and epithelium of the medial and lateral nasal processes, between E10.5 and E11.5) are viable, but lack most first branchial arch-derived structures, including the maxilla and the mandible (Trumpp et al., 1999). In addition, the level of *Fgf8* expression is strongly correlated with the phenotype in mutant mice: *Fgf8*$^{null/null}$ mice (0% of *Fgf8* expression level) die from defective gastrulation at an early embryonic stage (Sun et al., 1999); *Fgf8*$^{null/Neo}$ mutant mice (20% of *Fgf8* expression level) display midfacial cleft; *Fgf8*$^{Neo/Neo}$ newborn mice (40% of *Fgf8* expression level) exhibit altered nasal capsule and optic capsule, as well as trabecular basal plate; and *Fgf8*$^{+/null}$ and *Fgf8*$^{+/Neo}$ mice are phenotypically comparable to *Fgf8*$^{+/+}$ mice (50% of *Fgf8* expression level in *Fgf8*$^{+/null}$, 70% in *Fgf8*$^{+/Neo}$, and 100% in *Fgf8*$^{+/+}$ mice) (Griffin et al., 2013). These studies of mouse models demonstrate that FGF8 is a key mediator of proper orientation and polarity of facial primordia and subsequent frontonasal skeletal morphogenesis.

FGF Inhibitors—Sprouty (Spry) is one of the negative regulators of FGF signaling that plays crucial roles in embryogenesis (Hacohen et al., 1998). Mice with conditional expression of the *Spry1* transgene in CNC cells (*Spry1*^{TG}*;Wnt1-Cre* transgenic mice) exhibit midfacial cleft, cleft palate, and failure to form the nasal and frontal bones, as well as micrognathia and cardiovascular defects such as ventricular septal defects and outflow tract defects (Yang et al., 2010). In *Spry1*^{TG}*;Wnt1-Cre* transgenic mice, these defects appear to be the result of decreased proliferation and increased apoptosis of NC and NC-derived cell populations. The expression of transcription factors *Ap2* and *Msx2*, which are important to normal midfacial development, is reduced by inhibition of FGF signaling in *Spry1*^{TG}*;Wnt1-Cre* mice. By contrast, *Spry1*$^{-/-}$ and *Spry2*$^{-/-}$ mice display no midfacial developmental defect, but *Spry4*$^{-/-}$ mice exhibit midfacial hypoplasia, micrognathia, malocclusion, and increased

incisor size (Taniguchi et al., 2007). In addition, *Spry2*$^{-/-}$;*Spry4*$^{-/-}$ double knockout mice exhibit a more severe midfacial dysplasia than *Spry4*$^{-/-}$ mice (Taniguchi et al., 2007), suggesting that Spry2 and Spry4 have both a redundant and non-redundant function in FGF signaling during midfacial development.

2) WNT Signaling Pathway

WNT ligands are secreted glycoproteins that compose a large family of 19 proteins in humans. Different combinations of WNT ligands and receptors can activate at least three distinct signaling pathways: the β-catenin-dependent (canonical) pathway, the β-catenin-independent planar cell polarity (PCP) pathway, and the β-catenin-independent Ca^{2+} pathway. Canonical WNT signaling is mediated by β-catenin through its stability and translocation into the nucleus. Without WNT ligands, β-catenin is phosphorylated and degraded by a β-catenin destruction complex, which includes AXIN, adenomatous polyposis coli (APC), and glycogen synthase kinase 3 (GSK-3). In presence of WNT ligands, β-catenin accumulates in the cytoplasm and eventually translocates into the nucleus to act as a transcriptional co-activator of transcription factors that belong to the T-cell factor/lymphoid enhancing factor (TCF/LEF) family.

The WNT signaling pathway is a highly conserved pathway across multiple species and regulates crucial aspects of embryonic development (Komiya and Habas, 2008). In craniofacial development, WNT signaling plays critical roles in the generation, migration, and proliferation of CNC cells in the facial processes (Mani et al., 2010).

Upstream of WNT Signaling—Pre-B-cell leukemia homeobox (PBX) proteins are homeodomain-containing homeobox (HOX) cofactors that increase HOX DNA-binding specificity and are required for organogenesis and skeletogenesis (Capellini et al., 2006). At E8.5-9.0, *Pbx* genes (*Pbx1*, *Pbx2*, and *Pbx3*) are highly expressed within the frontonasal process and first branchial arch, as well as in the surface of cephalic ectoderm and mesenchyme at E9.0; at E10.5, *Pbx* genes are highly expressed in the epithelium of the maxillary process and medial and lateral nasal processes. PBX proteins can directly regulate gene expression of *Wnt9b* and *Wnt3*, which in turn act upstream of *Fgf8* (Ferretti et al., 2011). As a result, mice with a disrupted PBX pathway (*Pbx1*$^{-/-}$;*Pbx2*$^{+/-}$, *Pbx1*$^{F/F}$;*Pbx2*$^{+/-}$;*Foxg1-Cre* [*Foxg1-Cre* is expressed in facial and head ectoderm], and *Pbx1*$^{F/F}$;*Pbx2*$^{+/-}$;*Crect-Cre* [*Crect-Cre*, aka *Tcfap2a-Cre*, is induced by the ectoderm-specific *Tcfap2a* gene]) display cleft lip resulting from increased apoptosis at the medial edge of midfacial processes (Ferretti et al., 2011).

WNT Ligands—*Wnt9b*, a ligand for canonical WNT signaling, is expressed in the ectoderm of the facial processes and in the epithelial seam between the fusing facial processes during lip fusion in mice (Lan et al., 2006). *Wnt9b* null mice (*Wnt9b*$^{-/-}$ mice) show retarded outgrowth of the nasal (lateral and medial) and maxillary processes resulting from reduced proliferation of mesenchymal cells through tissue-tissue interactions, which subsequently leads to bilateral cleft lip and cleft palate (Jin et al., 2012). The cellular defects in *Wnt9b*$^{-/-}$ mice are mainly caused by reduced gene expression of members of the FGF family (Jin et al., 2012). As seen in *Wnt9b*$^{-/-}$ mice, deletion of *Wnt9b* in the cephalic

ectoderm region (*Wnt9b*$^{F/F}$*;Foxg1-Cre* mice) causes bilateral cleft lip through a mechanism similar to that of *Wnt9b*$^{-/-}$mice. In addition, compound mutant mice *Wnt9b*$^{-/-}$*;Pbx1*$^{+/-}$ (*Pbx1* is upstream of WNT signaling) and *Pbx1*$^{F/F}$*;Pbx2*$^{+/-}$*;Foxg1-Cre* mice both exhibit cleft lip (Ferretti et al., 2011). These studies strongly suggest that the PBX-WNT pathway plays a crucial role in the induction of apoptosis in the medial edge epithelium during midfacial fusion.

WNT Receptor—In the canonical WNT pathway, WNT ligands bind to a receptor complex comprising members of the Frizzled family and a coreceptor of the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). Mice with ablation of *Lrp6* (*Lrp6*$^{\beta geo/\beta geo}$ null mice) exhibit bilateral cleft lip, hypoplasia of the upper lip, cleft palate, and midline cleft of the mandible with complete penetrance (Song et al., 2009). Moreover, both proliferation and apoptosis are altered in *Lrp6*$^{\beta geo/\beta geo}$ lip epithelium, following downregulation of *Msx1* and *Msx2* expression.

WNT Signaling Mediators—AXIN (AXIN1 and AXIN2) proteins are negative regulators of canonical WNT signaling that promote the degradation of β-catenin (Zeng et al., 1997). Targeted disruption of *Axin2* (*Axin2*$^{-/-}$ mice) promotes osteoblast proliferation and differentiation and induces skull structure malformations, including midfacial hypoplasia (Yu et al., 2005). Loss of *Axin1* (*Axin1*$^{-/-}$) or dominant negative knock-in of *Axin1* allele, in which the regulator of G-protein signaling (RGS) domain or the six C-terminal amino acids (C6 motif) in *Axin1* are deleted (*Axin1*$^{\Delta RGS/\Delta RGS}$ and *Axin1*$^{\Delta C6/\Delta C6}$ mice), results in augmented (i.e., gain-of-function) β-catenin-dependent WNT signaling and early embryonic lethality at E9.5-E10.5 (Chia et al., 2009; Zeng et al., 1997). While the early embryonic lethality of *Axin1*$^{\Delta C6/\Delta C6}$ mutant mice is rescued by haploinsufficiency of β-catenin (*Ctnnb1*$^{+/-}$), *Axin1*$^{\Delta C6/\Delta C6}$*;Ctnnb1*$^{+/-}$ mice still die within one day after birth presenting midfacial cleft (Chia et al., 2009). Similarly, loss-of-function of β-catenin in CNC cells (*Ctnnb*$^{F/F}$*;Wnt1-Cre*) leads to severe craniofacial abnormalities in mice (Brault et al., 2001). Thus, precise levels of WNT/β-catenin signaling activity seem to be crucial for midfacial development.

Downstream Targets of WNT Signaling—The TCF/LEF family regulates gene expression of WNT/β-catenin signaling downstream targets (Cadigan, 2012). For example, the TCF1 protein regulates gene expression of *foxd3*, a transcriptional repressor of the winged helix or “Forkhead” family of proteins, during *Xenopus* gastrulation (Janssens et al., 2013). *Foxd3* is expressed in premigratory and migrating NC cells and is one of the earliest molecular markers of the NC lineage (Teng et al., 2008). Mice with a deficiency of *Foxd3* in CNC cells (*Foxd3*$^{F/-}$*;Wnt1-Cre* mice) die at birth with midfacial cleft (Teng et al., 2008).

Noncanonical WNT Signaling Pathway—Collagen triple helix repeat containing 1 (CTHRC1), a secreted glycoprotein, is involved in selective activation of the non-canonical PCP pathway induced by WNT ligands. The VANGL proteins, which are mammalian homologues of the *Drosophila* melanogaster protein Van Gogh (Vang/Strabismus) containing four transmembrane domains and intracellular N- and C- termini, can provide the scaffolding necessary for the assembly of PCP signaling complexes. Although *Cthrc1* null

mutant (*Cthrc1*$^{-/-}$) mice appear normal, the introduction of a heterozygous mutation in a PCP gene, *Vangl2* (*Cthrc1*$^{-/-}$;*Vangl2*$^{+/-}$), results in craniofacial deformities, including midfacial hypoplasia (Yamamoto et al., 2008). This indicates a compensative function of *Vangl2* on a *Cthrc1*$^{-/-}$ background and the importance of the PCP pathway in midfacial development.

3) Rho GTPase Signaling Pathway

Rho-related small GTPases (e.g., RhoA, Rac1, and Cdc42) have emerged as key mediators of the WNT signaling pathway, most notably in the non-canonical pathways, including the PCP pathway, but also in the canonical pathway. Rho GTPases transduce extracellular signals into a variety of intracellular events controlling cell morphology and polarity (Marinissen et al., 2004). Key Rho effectors Rho kinase 1 (*aka* ROCK1) and ROCK2 are highly conserved serine/threonine kinases with multiple substrates and play essential roles in fundamental cellular processes, such as contraction, adhesion, migration, apoptosis, and proliferation (Julian and Olson, 2014).

Rho Signaling Mediators—The expression patterns of *Rock1* and *Rock2* are similar in developing embryos (Wei et al., 2001). Mice expressing a dominant negative (DN) form of ROCKs in CNC cells (*Rock*$^{DN/DN}$;*Wnt1-Cre* mice) exhibit hypoplasia of the frontonasal processes and first branchial arch, ultimately resulting in hypoplasia of the maxilla and mandible, midfacial cleft, and hypertelorism (Phillips et al., 2012). CNC cells in *Rock*$^{DN/DN}$;*Wnt1-Cre* mice show exacerbated cell death in the frontonasal processes (Phillips et al., 2012). In humans, a deletion on chromosome 18q, where *ROCK1* resides (chromosome 18q deletion syndrome, OMIM 601808), causes a spectrum of abnormalities that include hypertelorism, cleft palate, micrognathia, and cardiac defects (Phillips et al., 2012).

Rho GTPases—*Cdc42* and *Rac1* are ubiquitously expressed small Rho GTPases that act as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound state. Mice with deletion of either *Cdc42* or *Rac1* in the NC (*Cdc42*$^{F/F}$;*Wnt1-Cre* and *Rac1*$^{F/F}$;*Wnt1-Cre*) exhibit craniofacial deformities such as midfacial cleft and cleft palate resulting from a reduction in size of NC-derived structures. Increased cell-cycle exit is detected in these bony structures, while NC cells emigrating from the neural tube are not affected (Fuchs et al., 2009; Liu et al., 2013). In addition to these phenotypes, epithelial detachment, formation of acellular mesenchymal cavities, and elevated apoptosis have been found in CNC-derived mesenchymal regions of *Rac1*$^{F/F}$;*Wnt1-Cre* mice (Thomas et al., 2010). In contrast to *Cdc42*$^{F/F}$;*Wnt1-Cre* and *Rac1*$^{F/F}$;*Wnt1-Cre* mice, *RhoA*$^{F/F}$;*Wnt1-Cre* mice exhibit exencephaly but no obvious midfacial defects (Katayama et al., 2011). Although there is no conclusive evidence explaining these craniofacial phenotypic differences, they may reflect differential expression and regulation of RhoA in craniofacial development.

4) SHH Signaling Pathway

Sonic hedgehog (SHH) is a member of the hedgehog (HH) family of secreted proteins and plays crucial roles in diverse developmental processes, including left-right axis establishment, dorsoventral patterning of the neural tube, endoderm development,

anteroposterior patterning of the developing limb, and brain development and patterning (Roessler and Muenke, 2003). The foregut endoderm, facial ectoderm, and neuroepithelium are all sources of SHH ligands (Echelard et al., 1993), which bind to their cell surface receptor Patched 1 (PTCH1), hence triggering SHH signaling, to relieve its basal repression of Smoothened (SMO) (Jiang et al., 2006; Pan et al., 2013). SHH signaling is critical for proliferation, survival, and patterning of CNC cells during craniofacial development (Hu and Marcucio, 2009; Iwata et al., 2014).

SHH Receptors—SHH ligands bind to PTCH1, a primary receptor for SHH ligands that works as a repressor of SHH signaling, in the absence of SHH ligand and co-receptors SMO, cell adhesion molecule-related/downregulated by oncogenes, *aka* CDO (CDON), brother of CDON (BOC), and growth arrest-specific 1 (GAS1) to induce SHH signaling activity. Mice with deletion of *Smoothened* in CNC cells ($Smo^{F/-}$*;Wnt1-Cre* mice) exhibit severe craniofacial deformities such as midfacial hypoplasia, hypoplastic nasal bone, cleft palate, and micrognathia, owing to increased apoptosis and decreased cell proliferation of postmigratory CNC cells (Jeong et al., 2004). $Cdon^{-/-}$ mice show variability in the penetrance and expressivity of midfacial and holoprosencephaly phenotypes (e.g., absence of primary palate, hypoplasia of nasal septum cartilage, absence of maxillary incisors) in a strain-dependent manner (Cole and Krauss, 2003; Zhang et al., 2006). In fact, genetic variation in different inbred strains of mice might be sufficient to account for these differences in penetrance and expressivity, as seen in human populations.

SHH Ligands—Mutations in human *SHH* are responsible for holoprosencephaly (HPE), a disorder involving abnormalities of the forebrain and facial structures (Belloni et al., 1996). *Shh* null mutant mice ($Shh^{-/-}$ mice) show early embryonic lethality with severe craniofacial deformities (Chiang et al., 1996), but mice with a single allele of an epithelial cell-specific deletion of *Shh* in a *Shh* heterozygous null background ($Shh^{c/n}$*;K14-Cre* mice), as well as mice with loss of *Shh* in the epithelium ($Shh^{F/F}$*;K14-Cre* mice), exhibit nasal hypoplasia and cleft palate (Iwata et al., 2014; Lan and Jiang, 2009). Similarly, excessive SHH signaling activity in *K14-Shh* transgenic mice causes hypertelorism, wide cleft lip (a failure of growth of the maxillary processes), cleft palate, absence of flat bones within the skull vault, and arrested tooth development with decreased cell proliferation (Cobourne et al., 2009). Importantly, SHH ligands are post-translationally modified with both cholesterol and palmitic acid, which are required for HH protein multimerization, distribution and activity. Disrupting post-translational palmitoylation of SHH ligands by inhibiting the hedgehog acyltransferase (*Hhat*) gene disturbs SHH secretion and long-range activity and causes severe hypotelorism with midfacial bone defects in $Hhat^{Creface/Creface}$ (The *Creface* [*Ap2-Cre*] transgene is integrated within intron 9 of *Hhat* to delete the *Hhat* gene) mice (Dennis et al., 2012). SHH-mediated WNT signaling activation is compromised in $Hhat^{Creface/Creface}$ mice during the growth and fusion of the nasal processes (Kurosaka et al., 2014). Since disruption of *Ptch1* results in an elevation of SHH signaling (Goodrich et al., 1997), severe midfacial defects in $Hhat^{Creface/Creface}$ mice are partially restored by a deletion of the *Ptch1* gene ($Hhat^{Creface/Creface}$*;*$Ptch1^{wiggable/wiggable}$ mice: $Ptch1^{wiggable}$ mice were generated through *N*-ethyl-*N*-nitrosourea [ENU] mutagenesis and the *Ptch1* gene is mutated), although $Hhat^{Creface/Creface}$*;*$Ptch1^{wiggable/wiggable}$ mice still exhibit hypoplastic nasal process

outgrowth, epithelial seam persistence, and cleft lip (Kurosaka et al., 2014). These studies indicate that both expression level and lipid modifications of SHH ligands are important for proper functioning of SHH ligands.

Primary Cilia—The primary cilium, a microtubule-based organelle, is required to induce HH signaling, a crucial pathway for embryonic patterning and organogenesis (Zaghloul and Brugmann, 2011). Two intraflagellar transport (IFT) complexes, IFT-A (6 subunits) and IFT-B (14 subunits), are required for the assembly and functioning of the primary cilia by mediating the activity of a number of developmental signaling pathways, including the HH, WNT, PDGF, FGF, and Notch cascades (Ashe et al., 2012; Liem et al., 2012). Of these signaling pathways, particularly HH signaling has been studied to date (Ashe et al., 2012; Brugmann et al., 2010).

In humans, mutations in *IFT144* and *IFT43* have been found in patients with Sensenbrenner and Jeune syndromes, which are characterized by craniofacial defects with hypertelorism, short ribs and limbs, and polydactyly, the result of increased SHH signaling (Arts et al., 2011; Ashe et al., 2012; Bredrup et al., 2011). Significantly, ENU-derived mutant mice [Random ENU mutagenesis is a complementary approach to obtain mouse models for human diseases (Nolan et al., 2000)] with a hypomorphic missense mutation in the *Ift144* gene ($Itf144^{twt}$ mice) exhibit skeletal and craniofacial anomalies, including facial cleft, frontonasal dysplasia, cleft palate, and exencephaly, as seen in patients with human skeletal ciliopathies (Ashe et al., 2012). Recent studies also show that elimination of the kinesin family member 3A (KIF3A), another IFT protein, in CNC cells ($Kif3a^{F/F}$*;Wnt1-Cre*) causes truncation of primary cilia on CNC cells and leads to expansion of the SHH-responsive area in the craniofacial mesenchyme (Brugmann et al., 2010; Liu et al., 2014). As a result, $Kif3a^{F/F}$*;Wnt1-Cre* mice exhibit hypertelorism, cleft lip, frontonasal dysplasia, and cleft palate. Thus, molecules related to cilia formation play crucial roles in regulating multiple signaling cascades in midfacial development.

5) TGFβ Superfamily Signaling Pathway

The members of the transforming growth factor beta (TGFβ) superfamily form dimers and bind to heterodimeric receptor complexes consisting of two type I (TGFBR1/ALK5) and two type II receptors (TGFBR2) with serine/threonine kinase domains. Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor and induces SMAD phosphorylation and translocation into the nucleus to regulate transcriptional activity of target genes. TGFβ signaling plays a critical role in a vast range of biological functions during craniofacial development, including cell proliferation, differentiation, and apoptosis (Iwata et al., 2011; Iwata et al., 2012).

Members of TGFβ signaling cascade are expressed in developing mouse facial primordia in a temporal and spatial specific manner (Dudas and Kaartinen, 2005). While the most established downstream signaling cascade involves the sequential phosphorylation of SMAD2/3 proteins (*aka* canonical pathway) in the formation of the frontal bone and some parts of other craniofacial structures, non-canonical (SMAD-independent) TGFβ signaling in

CNC cells plays a crucial role in the formation of the palate and the majority of the craniofacial structures (Ho et al., 2015; Iwata et al., 2011).

The signaling pathway of bone morphogenetic proteins (BMPs), members of the TGFβ superfamily, regulates diverse developmental processes, including cell proliferation, differentiation, apoptosis, and tissue morphogenesis (Wan and Cao, 2005). BMPs signal through receptor complexes composed of type II (BMPR2, ACVR2A, and ACVR2B) and type I transmembrane receptors (ACVR1A/ALK2, BMPR1A/ALK3, and BMPR1B/ALK6); binding of BMPs to their receptors induces SMAD1/5/8 phosphorylation and translocation into the nucleus to regulate gene expression of the targets. Members of the BMP family are essential for the development of the mammalian orofacial region, due to their role in the regulation of cell proliferation, extracellular matrix synthesis, and cellular differentiation. Perturbation of BMP signaling results in orofacial clefting.

TGFβ Receptors—Deletion of TGFβ receptor type I (*Tgfbr1*, aka *Alk5*) in CNC cells (*Alk5*$^{F/F}$*;Wnt1-Cre* mice) results in midfacial cleft with increased number of apoptotic cells within the mesenchyme of the first branchial arch (Dudas et al., 2006). Mice with loss of *Alk5* in the first branchial arch ectoderm (*Alk5*$^{F/F}$*;Nes-Cre* mice) die at E15 and display a hypoplastic maxillary process, unilateral or bilateral cleft lip, and cleft palate due to increased cell death in the medial nasal process and the maxillary process (Li et al., 2008). Interestingly, mice deficient for the other TGFβ receptors and ligands exhibit cleft palate, but no midfacial defects. This suggests that a combination of each ligand, receptor, and mediator differently activates a tissue-specific TGFβ cascade.

BMP Ligands—Embryonic orofacial tissues express BMPs and their receptors in a temporal and spatial specific manner, suggesting that BMPs play a crucial role in midfacial development. The role of BMP signaling is relatively well studied in the palate (Greene and Pisano, 2010; Nie et al., 2006b), but its involvement in midfacial development is still largely unknown. Mice with conditional inactivation of the BMP4 ligand (*Bmp4*) using the *Nes-Cre* transgenic line (*Bmp4*$^{F/F}$*;Nes-Cre* mice) exhibit isolated bilateral cleft lip at E12.5, and a few (2/9 mice) exhibit unilateral cleft lip at E14.5 (Liu et al., 2005). However, the mechanism of cleft lip in *Bmp4*$^{F/F}$*;Nes-Cre* mice is still largely unknown.

BMP Receptors—Mutations in BMP receptor type 1A (*BMPR1A*) are associated with facial dysmorphism in both juvenile polyposis and chromosome 10q23 deletion syndrome (Cao et al., 2006; Zhou et al., 2001). Mice harboring a DN form of *Bmpr1a* in NC-derived cells expressing the myelin protein zero (*Mpz-Cre*) transgene (*Bmpr1a*$^{DN/DN}$*;Mpz-Cre* mice) exhibit wide-open anterior fontanelles with complete penetrance as a result of activated p53-dependent apoptosis (Saito et al., 2012). A total of 80% of *Bmpr1a*$^{DN/DN}$*;Mpz-Cre* mice die from cleft face and palate soon after birth, and the other 20% survive and develop short faces, hypertelorism, and calvaria deformities.

BMP Inhibitors—The distribution and activity of BMPs are regulated by the antagonists Chordin (CHRD) and Noggin (NOG) in the extracellular space (Gazzerro and Canalis, 2006). *Noggin* null (*Nog*$^{-/-}$) mice show early midfacial narrowing in the spectrum of holoprosencephaly, which is a heterogeneous craniofacial and neural developmental

anomaly characterized in its most severe form by failure of the forebrain to divide (Lana-Elola et al., 2011). In addition, in *Nog*$^{-/-}$ mice, the expression area of *Shh*, as well as SHH target genes *Ptch1* and *Gli1*, is decreased in the frontonasal region at E11.5 and E12.5, which are key developmental stages of early facial development. *Chrd*$^{-/-}$ mice die perinatally with an extensive array of malformations, including midfacial deformities, cleft palate, and a small jaw (Bachiller et al., 2003). Although *Chrd*$^{-/-}$;*Nog*$^{-/-}$ double knockout mice show early lethality (*Chrd* and *Nog* are functionally redundant in embryogenesis), *Chrd*$^{-/-}$;*Nog*$^{+/-}$ mice survive until the neonatal stage and present defects restricted to the craniofacial region, with a varying degree of severity (but more severe than *Chrd*$^{-/-}$ single knockout mice) ranging from a single nostril with narrow proboscis, anterior truncation, agnathia, and midfacial hypoplasia to bilateral cleft lip (Anderson et al., 2002). Gene expression of *Shh* is also decreased in *Chrd*$^{-/-}$;*Nog*$^{+/-}$ mice, as seen in *Nog*$^{-/-}$ and *Chrd*$^{-/-}$ mice. Taken together, these findings indicate that BMP-mediated SHH signaling seems to play a crucial role in midfacial development.

6) PDGF Signaling Pathway

Platelet-derived growth factors (PDGFs) were originally identified as mitogenic factors for smooth muscle cells, fibroblasts, and glia cells. Taking into account recent findings, they are additionally known as paracrine growth factors that mediate epithelial-mesenchymal interactions in various tissues (Heldin and Westermark, 1999). PDGF ligands (PDGF-A to PDGF-D) exert their function by inducing dimerization and activation of the PDGF receptors (PDGFRα and PDGFRβ). Ligand binding leads to phosphorylation of tyrosine residues in their cytoplasmic domains, which in turn results in activation of a multitude of intracellular signaling cascades (Smith and Tallquist, 2010). PDGF signaling regulates progenitor cell proliferation and cell migration during early embryonic development and also controls extracellular matrix formation, tissue remodeling, and patterning determination during later stages of embryogenesis (Sun et al., 2000).

PDGF Receptors—PDGFRα is expressed in CNC cells and is required for their migration into the branchial arches (Smith and Tallquist, 2010). The *Patch* (*Ph*) mutation, originally identified as a distinctive pigmentation phenotype, includes a *PDGFRα* deletion. The homozygous null *Patch* mutation in mice (*Ph*$^{-/-}$ mice) results in pleiotropic defects and first branchial arch deformities, including facial clefting, hemifacial microsomia, micrognathia, opened neural tube, and heart deformities (Morrison-Graham et al., 1992; Gruneberg H and Truslove GM, 1960). *Pdgfra*-deficient mice (*Pdgfra*$^{-/-}$ mice) die by E15.5 and have a severe phenotype of midfacial cleft, spina bifida, subepidermal blistering, and skeletal and vascular defects (Soriano, 1997; Tallquist and Soriano, 2003). Similarly, loss of *Pdgfra* in CNC cells (*Pdgfra*$^{F/F}$;*Wnt1-Cre*) results in disruption of CNC cell migration and proliferation, which are essential to form the medial nasal processes as well as cranial bone and cartilage (He and Soriano, 2013). As a result, *Pdgfra*$^{F/F}$;*Wnt1-Cre* mice exhibit midfacial cleft, shortened nasal cartilage and premaxilla, and cleft palate.

PDGF Ligands—PDGF-A and PDGF-C are members of the PDGF family that signal through PDGF receptors. *Pdgfa*$^{-/-}$ mice exhibit no craniofacial defect, although *Pdgfc*$^{-/-}$ mice show cleft palate and subepidermal blood-filled blisters in the frontal and frontonasal

regions, resulting from diminished filopodia formation of epithelial cells and cell proliferation defects in both epithelium and mesenchyme (Ding et al., 2004). Interestingly, *Pdgfa*$^{-/-}$;*Pdgfc*$^{-/-}$ double knockout mice exhibit a midfacial cleft, spina bifida, subepidermal blistering, and skeletal and vascular defects, as seen in *Pdgfra*$^{-/-}$ mice (Ding et al., 2004). This suggests that both PDGF-A and PDGF-C signal through PDGFRα and that PDGF-C is dominant in the midfacial region, although it is in part functionally redundant with PDGF-A. These two genes also exhibit distinct expression patterns (He and Soriano, 2013).

7) Notch Signaling Pathway

The Notch signaling pathway is a cell-cell adhesion signaling mechanism evolutionarily conserved among species. A Notch transmembrane receptor interacts with a Notch transmembrane ligand on a contacting cell, initiating proteolytic cleavage of the receptor and the subsequent release of the Notch intracellular domain (NICD) of the receptor. The NICD then translocates into the nucleus to induce the transcription of Notch target genes. Several gene mutations of this pathway have been implicated in craniofacial birth defects.

Notch Ligands—Mutations in *JAGGED1* (*JAG1*), a ligand of the Notch signaling pathway, cause Alagille syndrome characterized by biliary, cardiac, and craniofacial anomalies, including a prominent forehead, deep-set eyes, a straight nose with a flat tip, flattened midface, and prominent chin. *Jag1*$^{F/F}$;*Wnt1-Cre* mice exhibit shortened maxillary regions and class III malocclusion but can survive until adulthood. The midfacial hypoplasia in *Jag1*$^{F/F}$;*Wnt1-Cre* mice results from decreased cell proliferation and continuous reduction in hyaluronic acid production at E14.5 and later stages (Humphreys et al., 2012). In the maxilla, cell autonomous *Jagged1* signaling in CNC-derived maxillary mesenchyme is required for osteoblast differentiation and mineralization (Hill et al., 2014).

8) MicroRNAs

MicroRNAs (miRs) are short (∼22 nucleotides) noncoding RNAs that regulate approximately 30% of protein-coding genes at the post-transcriptional level (Ross et al., 2007). miRs repress gene expression post-transcriptionally by Watson-Crick base pairing to the seed sites in the 3′ untranslated region (UTR) of target genes. Multiple processes are involved in the synthesis and transcription of miRs as long primary transcripts that are cleaved by Dicer, a type 3 ribonuclease, to produce mature miRs (Wilson and Doudna, 2013). Recent studies indicate that loss of all mature miRs in CNC cells (*Dicer*$^{F/F}$;*Wnt1-Cre*) in mice results in increased apoptosis and defects in osteogenic differentiation, causing severe craniofacial deformities including midfacial cleft and hypoplasia (Huang et al., 2010; Nie et al., 2011; Sheehy et al., 2010; Zehir et al., 2010). These findings suggest that miRs play crucial roles in the fate determination of CNC cells. By contrast, although *Dicer* was expected to play a role in palatal fusion, epithelial-specific *Dicer* knockout (*Dicer*$^{F/F}$;*K14-Cre*: *K14-Cre* is expressed after the fusion of the upper lip) mice exhibit no significant changes in craniofacial development (Otsuka-Tanaka et al., 2013). Future studies are thus necessary to understand the role of miRs during the fusion of facial processes.

miRs Associated with BMP Signaling—The *miR-17-92* cluster encodes six miRs (*miR-17*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a*, and *miR-92a-1*) within a region on

chromosome 13q, whose deletion is associated with cleft lip and/or palate, lung hypoplasia, microophthalmia, microcephaly, and small stature in humans (Kirchhoff et al., 2009). A recent study shows that the *MiR-17-92* cluster is a direct target of BMP signaling and transcription factor AP2α (Wang et al., 2013b). Mice with *miR-17-92* deficiency (*miR-17-92*$^{-/-}$ mice: the clefting phenotype is incompletely penetrant) and compound deletion of *miR-17-92* and the related *miR-106b-25* cluster (*miR-17-92*$^{-/-}$*;miR-106b-25*$^{-/+}$ mice: the clefting phenotype is more severe and completely penetrant) exhibit cleft lip, cleft palate, and mandibular hypoplasia owing to cell proliferation defects in the mesenchyme (Wang et al., 2013b).

9) Transcription Factors

ALX3/ALX4—Aristaless-like homeobox genes such as *Alx3* and *Alx4* form a distinct gene family, whose members are characterized by a paired type homeobox with partially redundant functions and a small conserved C-terminal domain. Although mice with deletion of *Alx3* (*Alx3*$^{-/-}$ mice) or *Alx4* (*Alx4*$^{-/-}$ mice) are indistinguishable in regard to midfacial development from wild-type control mice, *Alx3*/*Alx4* double knockout (*Alx3*$^{-/-}$*;Alx4*$^{-/-}$) mice show severe craniofacial abnormalities, among them midfacial cleft, following an increase in apoptosis in the outgrowing frontonasal process at E10.0 (Beverdam et al., 2001). This indicates that *Alx3* and *Alx4* are functionally redundant in midfacial development.

FOXC1—*Foxc1* is a member of the forkhead box winged helix transcription factor family and plays roles in a variety of tissue developmental processes. *Foxc1* null (*Foxc1*$^{-/-}$) mice display bony syngnathia, defects in maxillary and mandibular structures, and craniofacial muscular developmental defects (Inman et al., 2013). Interestingly, mice with haploinsufficiency of *Fgf8* in a *Foxc1*$^{-/-}$ background (*Foxc1*$^{-/-}$*;Fgf8*$^{+/-}$) resemble *Foxc1*$^{-/-}$ mice but have more severe frontonasal shortening and no externally visible mandible. This indicates that *Foxc1* and *Fgf8* function synergistically in frontonasal and mandibular development (Inman et al., 2013).

GRHL2—Mice with an ENU-induced nonsense mutation in the *grainyhead-like 2* (*Grhl2*) gene that encodes transcription factor GRHL2 (*Grhl2*$^{1Nisw/1Nisw}$ mutant mice) display midfacial cleft and hypoplasia, exencephaly, body wall closure defects, and defective optic fissure closure with complete penetrance (Pyrgaki et al., 2011). In *Grhl2*$^{1Nisw/1Nisw}$ mutant mice, expression of adhesion molecules is downregulated during neural fold fusion, suggesting that GRHL2 plays a crucial role in epithelial fusion.

MSX1—*Msx1*$^{-/-}$ mice die at birth and exhibit midfacial hypoplasia, small nasal bones, rounded shape of the frontal bones, cleft palate, and a small mandible (Satokata and Maas, 1994). Although *Msx2*$^{-/-}$ mice exhibit no midfacial deformities or very mild ones, *Msx1*$^{-/-}$*;Msx2*$^{-/-}$ mice exhibit more severe midfacial developmental defects than *Msx1*$^{-/-}$ mice (Ishii et al., 2005; Satokata et al., 2000), which suggests that MSX1 and MSX2 are functionally redundant in midfacial development.

SOX11—Transcription factor SOX11 is expressed transiently during embryogenesis in many tissues that undergo inductive remodeling (Hong and Saint-Jeannet, 2005).

Approximately 70% of all *Sox11*$^{-/-}$ embryos exhibit cleft lip (unilateral or bilateral) and cleft palate resulting from failure of the maxillary processes to fuse with the lateral nasal processes (Sock et al., 2004). Unfortunately, the cellular mechanism behind this phenotype has not been studied yet.

TCFAP2A—AP2α, a member of a small family of "basic-helix-span-helix" transcription factors, is a retinoic acid (RA)-inducible transcriptional factor expressed in epithelial and NC cell lineages during development (Knight et al., 2005; Zhang et al., 1996). Encoded by *Tcfap2a* in mice and *TFAP2A* in humans, AP2α is expressed in the developing midfacial region (Feng et al., 2008; Nelson and Williams, 2004). Mice with a homozygous disruption of *Tcfap2a* (*Tcfap2a*$^{-/-}$ mice) die at or before birth because of severe developmental defects in the neural tube, head, and body wall (Nottoli et al., 1998; Zhang et al., 1996). A total of 16% of chimeric null mice composed of both wild type and *Tcfap2a* null embryonic stem cells (*Tcfap2a*$^{Chi/Chi}$ mice) exhibit craniofacial dysmorphology, including cleft lip with or without cleft palate (Nottoli et al., 1998). The phenotypes observed in the chimeras display a significant overlap with those caused by teratogenic levels of RA (Nottoli et al., 1998). Altogether, these findings indicate that AP2α is an important component of the RA metabolism.

TWSG1—Mice with a deficiency of the twisted gastrulation gene (*Twsg1*$^{-/-}$ mice) with exposure to all-trans RA manifest HPE with 100% penetrance, compared to 17% without exposure to all-trans RA via induction of apoptosis following upregulated *Bmp2* expression and oxidative stress (Billington et al., 2015).

10) Others

INSIG1 and INSIG2—Insulin-induced genes 1 and 2 (Insig1/2), a pair of endoplasmic reticulum (ER) membrane proteins, are essential for end product-mediated feedback inhibition of cholesterol synthesis (Goldstein et al., 2006). Insig1/2 restricts the cholesterol biosynthetic pathway by preventing proteolytic activation of transcription factors sterol regulatory element-binding proteins (SREBPs) and by enhancing degradation of HMG-CoA reductase (Dong and Tang, 2010). Mice with loss of *Insig1/2* (*Insig1*$^{-/-}$;*Insig2*$^{-/-}$) die within one day after birth and present midfacial developmental defects ranging from cleft palate to complete midfacial cleft with hypoplastic processes of the midfacial region (Engelking et al., 2006). The liver and head of *Insig1*$^{-/-}$;*Insig2*$^{-/-}$ embryos overproduce sterols, causing a marked buildup of cholesterol intermediates. These findings have implications for the pathogenesis of midfacial cleft and other human malformation syndromes in which mutations in enzymes catalyzing the steps in cholesterol biosynthesis produce a buildup of sterol intermediates (Engelking et al., 2006). Although the cellular and molecular mechanism is still largely unknown, recent studies suggest that proper cholesterol metabolism is crucial for SHH signaling activation (Porter, 2006; Stottmann et al., 2011).

TCOF1—Autosomal dominant mutations of the *TCOF1* gene cause Treacher Collins syndrome, which is characterized by hypoplasia of the first branchial arch derivatives, including facial bones (particularly the zygomatic complex and the mandible), cleft palate, and middle and external ear defects (Dixon et al., 2007). Treacle, the protein encoded by

TCOF1, is required for the cell-autonomously formation and proliferation of NC cells (Dixon et al., 2006). Mice haploinsufficient for *Tcof1* (*Tcof1*$^{+/-}$ mice) exhibit frontonasal dysplasia and cleft palate as a result of increased apoptosis in neuroepithelial cells, a source of CNC cells (Dixon et al., 2006).

Spontaneous Mouse Models—The *Twirler (Tw)* mouse model has proven to be an excellent model in the study of midfacial development, with all *Tw* homozygotes exhibiting clefting of the midfacial region. The *Tw* mutation is semidominant, and all homozygous mice display either a unilateral or bilateral cleft lip with cleft palate and a disrupted nasal cavity with misshapen nasal turbinates that are reduced in size and number (Gong et al., 2000).

11) Environmental Risk Factors for Midfacial Developmental Defects

Environmental risk factors and nutritional deficiencies have been implicated in midfacial cleft and hypoplasia (Johnston and Bronsky, 1995; Krapels et al., 2004b). It has been shown that cigarette smoking, maternal alcohol consumption and abnormal diets (zinc, myoinositol, folic acid, RA, etc.) can cause midfacial defects (Krapels et al., 2004a; Mossey et al., 2009).

Fetal Alcohol Syndrome—Mouse models for fetal alcohol syndrome (treated with a high dosage of ethanol during pregnancy) show an anterior neural plate deficiency and cleft lip with increased cell death (Kotch and Sulik, 1992; Sulik, 2005). Disruption of the SHH signaling pathway has been implicated as an important molecular mechanism in the pathogenesis of fetal alcohol syndrome (Ahlgren et al., 2002; Kietzman et al., 2014). For example, a correlation between the severities of facial dysmorphology and ethanol exposure during pregnancy has been described in both *Shh*$^{+/-}$ and *Gli2*$^{+/-}$ mice (Kietzman et al., 2014). CDON, a cell surface SHH-binding protein, promotes SHH pathway activity as a co-receptor with PTCH1 (Sanchez-Arrones et al., 2012). *Cdon*$^{-/-}$ mice exposed to ethanol *in utero* exhibit more severe midfacial hypoplasia as well as a broad spectrum of holoprosencephaly phenotypes, compared to non-treated *Cdon*$^{-/-}$ mice (Hong and Krauss, 2012).

Retinoic Acid Metabolism—RA is derived from vitamin A (retinol) by retinal dehydrogenase. The first step of RA synthesis is the oxidation of retinol to retinal by alcohol dehydrogenases; subsequently, retinal is converted to RA by retinaldehyde dehydrogenases. Craniofacial dysmorphism has been observed in cases of disruption of RA synthesis, as well as of exposure to excessive RA (Johnston and Bronsky, 1995). RA originates from the ventral epithelium of the frontonasal process and targets CNC cells (Schneider et al., 2001). Mice with ENU-induced mutation in the retinol dehydrogenase 10 (*Rdh10*) gene (*Rdh10*$^{trex/trex}$ mutant mice) display craniofacial, eye, limb, and major organ abnormalities and die by approximately E13.0 (Sandell et al., 2007). *Rdh10*$^{-/-}$ mice rescued by maternal administration of the missing retinoid compound can survive postnatally (Rhinn et al., 2011). Deletion of the *Raldh2* gene, which is involved in the second step of RA synthesis, in mice causes lethality at E10.5. Treatment with RA can extend survival until E18.5, and these surviving embryos show midfacial hypoplasia (Niederreither et al., 2002). *Raldh3* null mice, on the other hand, present malformations restricted to the ocular and nasal regions, which

cause choanal atresia (aka upper airway obstruction) following downregulation of *Fgf8* in nasal fins (Dupe et al., 2003). Thus, RA metabolic defects at each step of synthesis cause midfacial birth defects.

Perspectives—Midfacial development involves a few morphogenic processes such as growth and fusion that are more complex than general morphogenic processes involved in the timing and balance of growth of midfacial primordia. This is well reflected by the fact that the etiology of midfacial birth defects is complicated by a variety of genetic and environmental risk factors, as listed in Table 1. During midfacial development, the size and pattern of the primordia dramatically change in a short time window. The expression of hundreds of genes changes dramatically at each embryonic stage during craniofacial development, as confirmed by global gene expression studies, including microarrays and RNA-seq. These changes may be regulated by extracellular cues such as growth factors and the consequent orchestrated mechanisms that ultimately regulate cell fate (proliferation, differentiation, apoptosis, etc.). Specifically, in this review we learned that the BMP-SHH-WNT-FGF signaling axis may play a crucial role in midfacial development although each cell signaling cascade may directly regulate gene expression of causative genes of midfacial developmental defects. In addition to gene mutations, environmental and epigenetic factors also affect the expression and phenotypes of hundreds of genes without altering the DNA sequence and cause a number of diseases. Recent studies suggest that the distribution and contribution of miRs, which are regulated by environmental factors, is altered in midfacial birth defects. Interestingly, some cellular metabolisms play crucial roles in midfacial development through the regulation of cell signaling activity, which constitutes another example of how environmental and epigenetic factors affect midfacial morphogenesis. As implicated in a wide range of physiological and pathological conditions, a key cell signaling pathway may be activated to regulate gene and miR expression in a temporal and spatial specific manner during midfacial development. Further genetic studies and animal models will advance our understanding of the roles of genetic and epigenetic factors in midfacial development and will hopefully enable us to design future therapeutic approaches to diagnose and prevent midfacial developmental defects.

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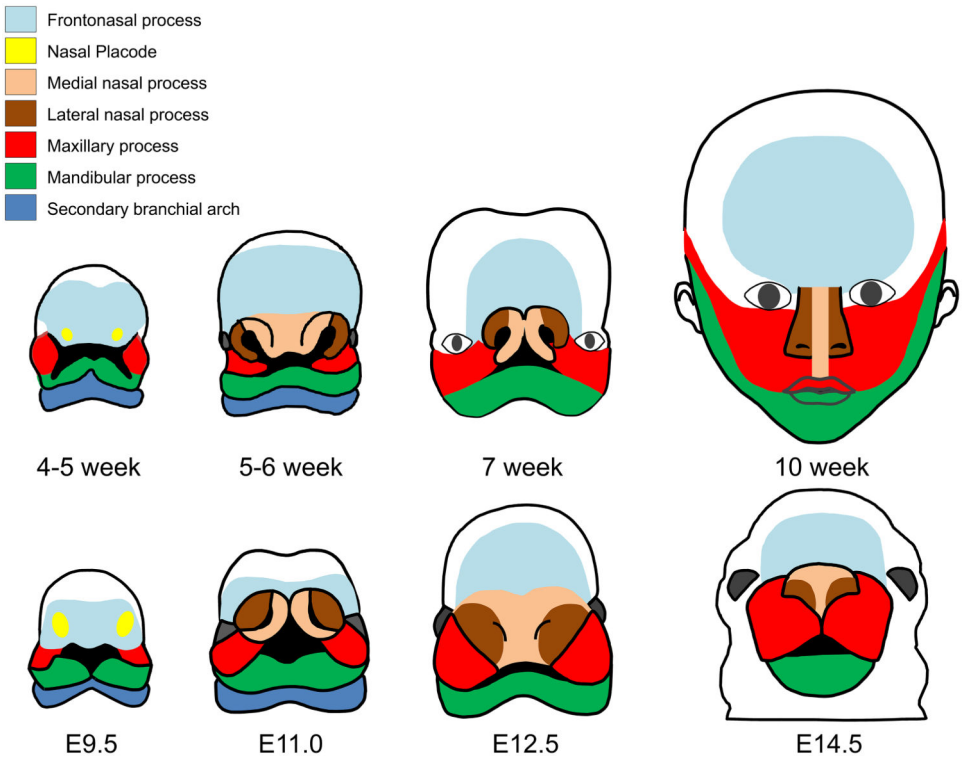

Fig. 1.

Development of the midfacial primordium. Frontal views of the prominences that give rise to the main structures of the face in humans (upper panels) and mice (lower panels), namely the frontonasal process (light blue), the nasal placode (yellow), the medial nasal processes (light orange), the lateral nasal processes (brown), the maxillary processes (red), the mandibular process (green), and the secondary branchial arch (blue).

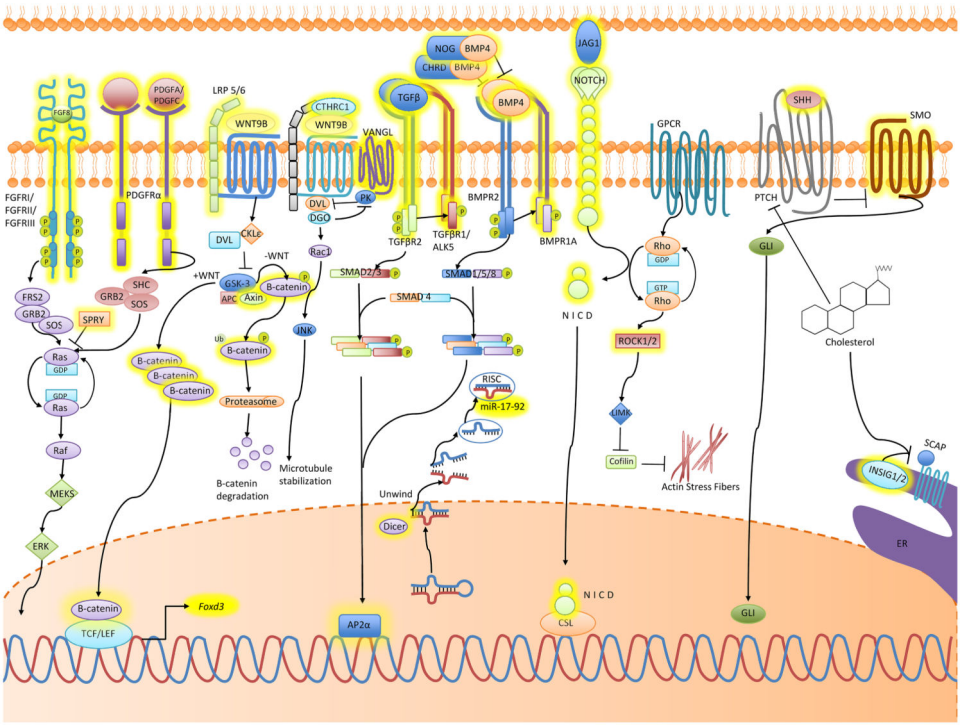

Fig. 2.

Cell signaling in midfacial development. Depicted FGF, PDGF, WNT, TGFβ, BMP, NOTCH, Rho, and SHH signaling cascades. Molecules that have been shown to play direct roles in midfacial deformities are highlighted in yellow, but we cannot exclude the possibility of the molecules not highlighted being important in midfacial development because these may not have yet been reported or they may be compensated by other molecules. CSL: CBF1/Su(H)/Lag-1 transcription factor complex, ER: endoplasmic reticulum, ERK: extracellular signal-related kinase, GPRC: G protein-coupled receptor, JNK: Jun amino-terminal kinases, LIMK: LIM domain kinase, MAPK: mitogen-activated protein kinase, MEKS: MAPK/ERK kinases, NICD: Notch intracellular domain, RISC: RNA-induced silencing complex, SCAP: sterol regulatory element-binding protein cleavage-activating protein.

Table 1

Selected mouse strains exhibited midfacial developmental defects.

Mouse Strain	Pathway/Component	Reference	Midfacial Phenotypes	Other Craniofacial Phenotypes
$Sp8^{-/-}$	Upstream of FGF signaling	(Kasberg et al., 2013)	Midfacial cleft, maxillary hypoplasia, hypertelorism	Exencephaly, absence of frontal and parietal bones
$Sp8^{F/F}$;Pax3-Cre	Upstream of FGF signaling	(Kasberg et al., 2013)	Midfacial cleft	Exencephaly, absence of frontal and parietal bones
$Sp8^{F/F}$;Foxg1-Cre	Upstream of FGF signaling	(Kasberg et al., 2013)	Midfacial cleft	Exencephaly, absence of frontal and parietal bones
$Fgfr1^{F/F}$;Wnt1-Cre	FGF receptor	(Wang et al., 2013a)	Cleft lip, premaxilla hypoplasia	Cleft palate, reduced frontonasal prominence and maxillary processes, micrognathia, tooth bud defect
$Fgfr2^{+/P253R}$	FGF receptor	(Wang et al., 2010)	Midfacial cleft and hypoplasia	Cleft palate, skeletal system malformation (skull, palate, sternum, and long bones), craniosynostosis
$Fgfr2^{+/S252W}$	FGF receptor	(Yu et al., 2000)	Midfacial cleft and hypoplasia	Skeletal system malformation, craniosynostosis
$Fgf8^{null/Neo}$	FGF ligand	(Griffin et al., 2013)	Midfacial cleft	Cleft palate
$Fgf8^{F/F}$;Nes-Cre	FGF ligand	(Trumpp et al., 1999)	Maxillomandibular malformation	Agnathia
$Spry1^{TG}$;Wnt1-Cre	FGF inhibitor	(Yang et al., 2010)	Midfacial cleft, malformed maxilla	Cleft palate, failure of nasal and frontal bone formation, micrognathia
$Spry4^{-/-}$	FGF inhibitor	(Taniguchi et al., 2007)	Midfacial hypoplasia	Micrognathia, malocclusion, increased incisors
$Spry2^{-/-}$;$Spry4^{-/-}$	FGF inhibitor	(Taniguchi et al., 2007)	Midfacial dysplasia	Micrognathia, malocclusion, increased incisors
$Pbx1^{-/-}$;$Pbx2^{+/-}$	Upstream of WNT signaling	(Ferretti et al., 2011)	Cleft lip (unilateral or bilateral), jaw hypoplasia	Cleft palate
$Pbx1^{F/F}$;$Pbx2^{+/-}$;Foxg1-Cre	Upstream of WNT signaling	(Ferretti et al., 2011)	Cleft lip (bilateral)	Abnormal nasal capsule, palatal and palatine processes of maxilla, absence of premaxillary process
$Pbx1^{F/F}$;$Pbx2^{+/-}$;Crect-Cre	Upstream of WNT signaling	(Ferretti et al., 2011)	Cleft lip (bilateral)	None
$Wnt9b^{-/-}$	WNT ligands	(Jin et al., 2012)	Cleft lip (bilateral)	Cleft palate
$Wnt9b^{-/-}$;$Pbx1^{+/-}$	WNT ligands	(Ferretti et al., 2011)	Cleft lip (bilateral)	Cleft palate
$Wnt9b^{F/F}$;Foxg1-Cre	WNT ligands	(Ferretti et al., 2011)	Cleft lip (bilateral)	Cleft palate
$Lrp6^{\beta geo/\beta geo}$	WNT receptor	(Song et al., 2009)	Cleft lip (bilateral), midfacial hypoplasia	Defects in neural tube, eye, brain, limb and bone, cleft palate, midline cleft of the mandible
$Axin1^{\Delta C6/\Delta C6}$;$Ctnnb1^{+/-}$	WNT signaling mediator	(Chia et al., 2009)	Midfacial cleft, absence of nasal structures	Multiple brain malformations
$Foxd3^{F/-}$;Wnt1-Cre	Downstream target of WNT signaling	(Teng et al., 2008)	Midfacial cleft, absence of frontal bone and nasal capsule	Small interparietal, parietal, basal occipital bones, and premaxilla, shortened and thickened mandible

Mouse Strain	Pathway/Component	Reference	Midfacial Phenotypes	Other Craniofacial Phenotypes
Cthrc1$^{-/-}$;Vangl2$^{+/-}$	WNT inhibitor	(Yamamoto et al., 2008)	Midfacial hypoplasia	Exencephaly, neural tube closure defect in midbrain
Shh$^{-/-}$	SHH ligand	(Chiang et al., 1996)	Severe craniofacial deformities, holoprosencephaly	Single optic vesicle, single nasal pit w/in a single olfactory placode
Shh$^{c/n}$;K14-Cre	SHH ligand	(Lan and Jiang, 2009)	Nasal hypoplasia	Cleft palate
Shh$^{F/F}$;K14-Cre	SHH ligand	(Iwata et al., 2014)	Nasal hypoplasia	Cleft palate
K14-Shh transgenic	SHH ligand	(Cobourne et al., 2009)	Cleft lip, hypertelorism	Cleft palate, arrested tooth development, absence of flat bones in skull vault, prominence of the frontal region, skeletal open bite, glossoptosis
Smo$^{F/-}$;Wnt1-Cre	SHH receptor	(Jeong et al., 2004)	Midfacial deformities	Hypoplastic nasal bone, incomplete nasal septum, absence of lacrimal bone, mesethmoid and vomer
Cdon$^{-/-}$	SHH receptor	(Cole and Krauss, 2003; Zhang et al., 2006)	Absence of primary palate	Hypoplasia of nasal septum cartilage, lack of maxillary incisors
Hhat$^{Creface/Creface}$	SHH ligand modifier	(Dennis et al., 2012)	Midfacial hypoplasia	Brain and eye abnormalities, acrania, agnathia
Hhat$^{Creface/Creface}$;Ptch1$^{wiggable/wiggable}$	SHH ligand modifier/ SHH receptor	(Kurosaka et al., 2014)	Cleft lip, hypoplastic nasal process	Epithelial seam persistence
Ift144^{twt}	SHH-Primary cilia	(Ashe et al., 2012)	Cleft lip (bilateral)	Exencephaly, skeletal and craniofacial anomalies
Kif3a$^{F/F}$;Wnt1-Cre	SHH-Primary cilia	(Brugmann et al., 2010; Liu et al., 2014)	Midfacial deformities	Cleft palate, brain abnormalities, craniosynostosis, hypertelorism
Alk5$^{F/F}$;Wnt1-Cre	TGFβ receptor	(Dudas et al., 2006)	Midfacial cleft, nasal cartilage cleft, hypoplastic premaxilla	Absence of frontal bones, cleft palate, reduced mandible
Alk5$^{F/F}$;Nes-Cre	TGFβ receptor	(Li et al., 2008)	Cleft lip (unilateral or bilateral), hypoplastic maxillary process	Cleft palate
Bmpr1a$^{DN/DN}$;Mpz-Cre	BMP receptor	(Saito et al., 2012)	Midfacial cleft	Hypertelorism, calvaria deformities, short faces, cleft palate, wide-open anterior fontanelles
Bmp4$^{F/F}$;Nes-Cre	BMP ligand	(Liu et al., 2005)	Cleft lip (unilateral or bilateral)	Cleft palate
Nog$^{-/-}$	BMP inhibitor	(Lana-Elola et al., 2011)	Enlarged nasal septum, holoprosencephaly	A solitary median maxillary incisor, larger hyoid bone, and absence of vomeronasal cartilage
Chrd$^{-/-}$;Nog$^{+/-}$	BMP inhibitor	(Anderson et al., 2002)	Midfacial hypoplasia, cleft lip (bilateral), single nostril with narrow proboscis	Agnathia, cebocephaly with cyclopic eye beneath proboscis
Ph$^{-/-}$	PDGF receptor	(Morrison-Graham et al., 1992)	Midfacial cleft	Hemifacial microsomia, micrognathia, opened neural tube
Pdgfra$^{-/-}$	PDGF receptor	(Soriano, 1997; Tallquist and	Midfacial cleft	Hypoplastic brachial arches, wavy neural tube, skeletal defects

Mouse Strain	Pathway/Component	Reference	Midfacial Phenotypes	Other Craniofacial Phenotypes
		Soriano, 2003)		
$Pdgfra^{F/F}$;Wnt1-Cre	PDGF receptor	(He and Soriano, 2013)	Midfacial cleft, absence of primary palate and philtrum	Shortening of nasal cartilage and premaxilla
$Pdgfa^{-/-}$;$Pdgfc^{-/-}$	PDGF ligand	(Ding et al., 2004)	Midfacial cleft	Loss of frontonasal tissues, open neural arches
$Jag1^{F/F}$;Wnt1-Cre	Notch ligand	(Hill et al., 2014; Humphreys et al., 2012)	Short maxillary regions, midfacial hypoplasia	Malocclusion
$Rock^{DN/DN}$;Wnt1-Cre	Rho signaling mediator	(Phillips et al., 2012)	Hypoplasia of maxilla, midfacial cleft	Hypertelorism, hypoplasia of mandible, absence of frontonasal bones
$Cdc42^{F/F}$;Wnt1-Cre	Rho GTPase	(Liu et al., 2013)	Cleft lip (unilateral or bilateral), midfacial hypoplasia, short snouts	Cleft palate, enlarged frontal cortex, encephalocele, micrognathia
$Rac1^{F/F}$;Wnt1-Cre	Rho GTPase	(Fuchs et al., 2009)	Cleft lip (unilateral or bilateral), midfacial hypoplasia	Cleft palate, enlarged frontal cortex, short snouts, encephalocele, micrognathia
$Dicer^{F/F}$;Wnt1-Cre	MicroRNA	(Huang et al., 2010; Nie et al., 2011; Sheehy et al., 2010; Zehir et al., 2010)	Midfacial cleft and hypoplasia, short nose, microsomia	Malformation of midbrain, cerebellum and mandible, cleft palate
miR-17-$92^{-/-}$	MicroRNA	(Wang et al., 2013b)	Cleft lip	Mandibular hypoplasia, incompletely penetrant cleft palate, microphthalmia
miR-17-$92^{+/-}$;miR-106b-$25^{+/-}$	MicroRNA	(Wang et al., 2013b)	Cleft lip	Mandibular hypoplasia, completely penetrant cleft palate
$Alx3^{-/-}$;$Alx4^{-/-}$	Transcription factor	(Beverdam et al., 2001)	Midfacial cleft and hypoplasia, split nose	Eye abnormalities, small skull vault
$Foxc1^{-/-}$	Transcription factor	(Inman et al., 2013)	Maxillomandibular malformation, bony syngnathia	Alisphenoid and squamosal hypoplasia
$Foxc1^{-/-}$;$Fgf8^{+/-}$	Transcription factor/ FGF ligand	(Inman et al., 2013)	Maxillary hypoplasia, severe syngnathia	Alisphenoid and squamosal hypoplasia
$Grhl2^{1Nisw/1Nisw}$	Transcription factor	(Pyrgaki et al., 2011)	Midfacial cleft and hypoplasia	Exencephaly, defective optic fissure closure with complete penetrance, absence of skeletal components of the top of the skull and midface
$Msx1^{-/-}$	Transcription factor	(Satokata and Maas, 1994)	Midfacial hypoplasia, small nasal bones, rounded shape of the frontal bones	Cleft palate, small mandible
$Msx1^{-/-}$;$Msx2^{-/-}$	Transcription factor	(Ishii et al., 2005; Satokata et al., 2000)	Defects in the fusion of the median lateral, nasal and maxillary prominences, midfacial hypoplasia	Exencephaly, cleft mandible, micrognathia
$Sox11^{-/-}$	Transcription factor	(Sock et al., 2004)	Cleft lip (unilateral or bilateral) and upper jaw, maxillary hypoplasia	Cleft palate, eyelid closure defect
$Tcfap2a^{-/-}$	Transcription factor	(Nottoli et al., 1998; Zhang et al., 1996)	Cleft lip, midline clefting of primary palate	Developmental defects in neural tube, head, and body wall, cleft palate

Mouse Strain	Pathway/Component	Reference	Midfacial Phenotypes	Other Craniofacial Phenotypes
Tcfap2a$^{Chi/Chi}$	Transcription factor	(Nottoli et al., 1998)	Cleft lip, maxillary hypoplasia	Cleft palate, exencephaly, malocclusion
Tcof1$^{+/-}$	Treacher Collins syndrome	(Dixon et al., 2006)	Maxillary hypoplasia, frontonasal dysplasia	Cleft palate, exencephaly, hypoplasia of the premaxilla, mandible, and temporal bones
Tw/Tw	Spontaneous	(Gong et al., 2000)	Cleft lip (unilateral or bilateral)	Cleft palate
Rdh10$^{trex/trex}$	RA metabolism	(Rhinn et al., 2011; Sandell et al., 2007)	Midfacial cleft	Eye abnormalities, micrognathia
Insig1$^{-/-}$;Insig2$^{-/-}$	Cholesterol metabolism	(Engelking et al., 2006)	Midfacial cleft and hypoplasia	Cleft palate, exencephaly, unilateral or bilateral microphthalmia (or anophthalmia)