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Planar cell polarity signaling in craniofacial development

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Out of the several signaling pathways controlling craniofacial development, the role of planar cell polarity (PCP) signaling is relatively poorly understood. This pathway, originally identified as a mechanism to maintain cell polarity within the epithelial cells of the Drosophila wing, has been linked to the proper development of a wide variety of tissues in vertebrates and invertebrates. While many of the pathway members are conserved, it appears that some of the members of the pathway act in a tissue-specific manner. Here, we discuss the role of this pathway in vertebrate craniofacial development, highlighting cranial neural crest migration, skull and palate formation and the role of non-traditional modulators of PCP signaling within this developmental process.

Introduction

Despite a variety of different facial features, the basic process of craniofacial formation, a key step in vertebrates' evolution, is remarkably similar.[1,2] Craniofacial development begins with the delamination and migration of the cranial neural crest cells (NCC). These cells migrate out of the dorsal neural tube and condense to form the pharyngeal skeleton and neurocranium.[2] The skull forms as a result of the replacement of the initial cartilaginous skeleton by bone and direct membranous ossification. In mammals, formation of the secondary palate requires a sequence of complex morphogenetic tissue movements. Numerous developmental signals have been linked to these processes, one of which is the Wnt/planar cell polarity (PCP) pathway. The role of this pathway was first identified in establishing polarity within the epithelial cells of the Drosophila wing. In vertebrates, the Wnt/PCP pathway has been linked to a variety of developmental processes, including the orientation of the sensory hair cells in the mammalian ear, and controls the narrowing of the mediolateral axis and the elongation of the anterior posterior axis during gastrulation (referred to as convergent extension in Xenopus, or convergence and extension in zebrafish).[3] In this review, we will discuss the role of Wnt/PCP pathway proteins involved in the critical stages of craniofacial development.

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Cranial Neural Crest Migration

Many of the core Wnt/PCP signaling proteins are expressed in the cranial neural crest cells[4-7] and are required for its proper migration.[8,9] Here, we will briefly discuss this role; for a more detailed review, see Clay and Halloran.[10]

Studies in zebrafish and Xenopus suggest that Wnt/PCP restricts lamellipodial protrusions to the leading edge of the cells during NCC migration. This signaling pathway interacts with fibronectin-stimulated syndecan 4 (sdc4), a transmembrane proteoglycan expressed in neural crest cells, which acts to inhibit rac, a small GTPase at the trailing edge of the cells.[11] As rhoA, a downstream effector of the PCP pathway, also inhibits rac, sdc4 acts in a parallel pathway to regulate neural crest migration. The localization of PCP signaling elements, such as wnt11, frizzled 7 (fzd7) and dishevelled (dsh), with cell-cell contact is important for inhibition of NCC locomotion. When NCCs come in contact with each other, they change direction and retract their protrusions, a process that requires proper PCP signaling.[8] Transmembrane molecules such as protein tyrosine kinase 7 (ptk7) are thought to mediate the migration of NCCs through the activation of the PCP pathway. In Xenopus, ptk7 is thought to form a complex with fzd7 that recruits dsh to the membrane.[12]

Role of Wnt/PCP Signaling in Skull Formation

Wnt signaling plays an important role in skeleton formation, and multiple groups have demonstrated that Wnt/PCP signaling is required for proper craniofacial development.[13-16] Of the many Wnt molecules currently studied, Wnt5 ligands seem to be critical for craniofacial development. There are two known *wnt5* genes in vertebrates, *wnt5a* and *wnt5b*.[17-19] Interestingly, in mammals, *Wnt5a* is associated with craniofacial development, whereas in teleost, the *wnt5b* homolog is critical.[20,21] Overexpression or a lack of *Wnt5a* results in a developmental delay of chondrocytes transitioning from proliferative to hypertrophic chondrocytes. Wnt5a signaling has been shown to regulate expression of type II collagen, upregulate c-Jun expression and activate the JNK-pathway, depending on the cartilage element assayed.[22,23]

While Wnt5 ligands have been classified as "non-canonical," the role they play in PCP signaling is controversial, as they can affect multiple Wnt receptor pathways, such as Frizzleds and

Ror2.[16] The Ror protein family is highly evolutionarily conserved from *Caenorhabditis elegans* to humans and consists of an extracellular Frizzled-like, cysteine-rich domain, which can bind directly to Wnt5a, and a cytoplasmic tyrosine kinase domain that can be hyperphosphorylated.[24-26] Expression of *Ror2* and *Wnt5a* have been detected in the developing mouse craniofacial cartilage and teeth.[19,27] While the *Ror2* mouse mutant has a shortened snout and a cleft palate[28-31] and *Wnt5a* mutant mice display hypertelorism, micrognathia and a triangular mouth,[15] a direct tie of these two genes with PCP signaling has yet to be identified in craniofacial cartilage. However, recent reports on limb formation have genetically and physically linked *Wnt5a*/*Ror2* to *van Gogh-like 2* (*Vangl2*) and PCP signaling.[24,32] Previous research has shown that the *Vangl2*$^{-/-}$ mouse has digit and claw defects very similar to those observed in humans with mutations in *WNT5A* and *ROR2*, which lead to a rare form of short-limbed dwarfism called Robinow syndrome and brachydactyly type B.[15,31,33-35] To identify whether *Wnt5a* and *Ror2* mutations modulate the *Vangl2* phenotype, double heterozygous and homozygous *Wnt5a; Vangl2* and *Ror2; Vangl2* mice were generated.[24,32] While the *Vangl2*$^{-/-}$; *Wnt5a*$^{+/-}$ mouse had shorter and wider digits and a stronger long-bone phenotype than the *Vangl2* mutant alone, the *Vangl2*$^{-/-}$; *Ror2*$^{-/-}$ was even more severe and was similar to that of the *Wnt5a* mutant. The loss of both *Vangl2* and *Ror2* lead to an increase in Wnt/β-catenin signaling in the limb, a result of the loss of *Wnt5a* repression on the canonical pathway. Co-immunoprecipitation and FRET analysis revealed that VANGL2 and ROR2 directly interact in the cytoplasm, where WNT5A-activated ROR2 receptor mediates VANGL2's phosphorylation. These results from the *Vangl2*$^{-/-}$; *Ror2*$^{-/-}$ mouse leads to a model where the WNT5A gradient sets up distinct levels of Vangl2 phosphorylation in the cytoplasm, which is required for proper limb formation. As all three of these molecules are expressed during vertebrate craniofacial development, it suggests a possible role for them during craniofacial development.[24]

Non-Traditional Modulators of Wnt/PCP Signaling in Cartilage and Bone Formation

Several studies have found mutations in glycoproteins, and the genes associated with their processing and targeting to the plasma membrane produce congenital defects reminiscent of Wnt/PCP mutants. Glypicans, extracellular proteins that are found throughout the animal kingdom, are composed of a cysteine-rich globular protein core, GPI anchor and heparan sulfate (HS) side chains located close to the plasma membrane. The HS side chains allow for glypicans to interact with a multitude of signaling molecules.[36,37] A zebrafish mutant in the *glypican 4* (*gpc4*) gene was first identified due to its compressed anterior-posterior body axis caused by a reduction in Wnt/PCP signaling that is required for convergence and extension movement of cells during gastrulation and that results in late embryonic lethality.[13,38] A closer examination of the *gpc4* mutant revealed shortened cartilages of the pharyngeal and neurocranium skeleton due to an inability of the chondrocytes to elongate and intercalate into a stacked cartilage element.[13,14] To better understand the role of Wnt/PCP signaling in cartilage and bone formation in *gpc4*-deficient zebrafish embryos, the lethal gastrulation defect was suppressed with the addition of *gpc4* mRNA.[14] This allowed observations of the larval and adult role of *gpc4* in skull formation and identified a persistent loss of stacked chondrocyte organization in both juvenile and adult mutants. Interestingly, the early larva disorganization of cartilage elements resulted in the loss of particular facial bones, such as the symplectic, as a consequence of an expansion of neighboring ossification centers. These studies demonstrate a clear role for *gpc4* and likely Wnt/PCP in the formation of craniofacial cartilages and subsequent skull ossification.

Two zebrafish mutants in *exostosin* genes (*ext1* and *2*) encoding glycosyltransferases and *papst1*, a 3'-phosphoadenosine 5'-phosphosulfate (PAPS) transporter, also exhibit severe craniofacial cartilage defects.[39] This provides further evidence that glypicans and their HS side chains play a critical role in PCP signaling, as both of these genes allow for the proper posttranslational modification of proteoglycans in the Golgi. Loss of these genes results in shorter cartilage elements composed of rounded disorganized cells instead of the thin elongated stacked chondrocytes, defects very similar to the phenotype seen in *wnt5b* and *gpc4* mutants.[13,21] Interestingly, mutations in human *EXT1* and *EXT2* genes were found to be the cause of hereditary multiple exostoses, a disease in which patients develop benign long-bone tumors during childhood.[40-42] In addition, the *Ext1* mice mutants display smaller craniofacial structures,[43] suggesting a role of EXTs in mammalian craniofacial development.

Another possible modulator of Wnt/PCP signaling via proteoglycans is *r-spondin 3* (*rspo3*), a member of the secreted protein family, which was once thought to only interact with Wnt/β-catenin signaling[44,45] but now has been shown to be important in the Wnt/PCP pathway. In a search for r-spondin receptors, rspo3 was found to bind specifically to cells expressing *gpc3* and *sdc4*, both known proteoglycans that interact with Wnt signaling, but was unable to bind to cells expressing *lrp6, kermen1* or *frizzled5*, all well-established elements of Wnt/β-catenin signaling.[46] To prove that *rspo3* and *sdc4* are part of the Wnt/PCP pathway, morpholinos (MO) against *rspo3* were targeted to different germ layers of Xenopus embryos. Not only did *rspo3* MO disrupt the morphogenetic process of convergent extension movements during gastrulation without disrupting the specification of mesoderm, it also disrupted the intercalation and stacking process of craniofacial cartilage, both phenotypes very reminiscent of the zebrafish Wnt/PCP mutant *gpc4*.[13,46] Interestingly, *rspo3* and *wnt5a* are required for *sdc4* PCP signaling via a clathrin-mediated endocytosis process, suggesting an important role for endocytosis in the control of Wnt/PCP signaling (**Fig. 1**).[46]

Mutations in genes associated with the secretion of signaling proteins result in disorganized craniofacial phenotypes similar to those discussed above, suggesting a potential role in PCP signaling. Two genes that encode proteins involved in this process in humans and zebrafish are *sec23a* and *sec24d*.[47-49] Both of these genes are part of the COPII complex, which transports newly translated proteins, such as Col2a1 and proteoglycans, from the endoplasmic reticulum to the Golgi, where posttranslational

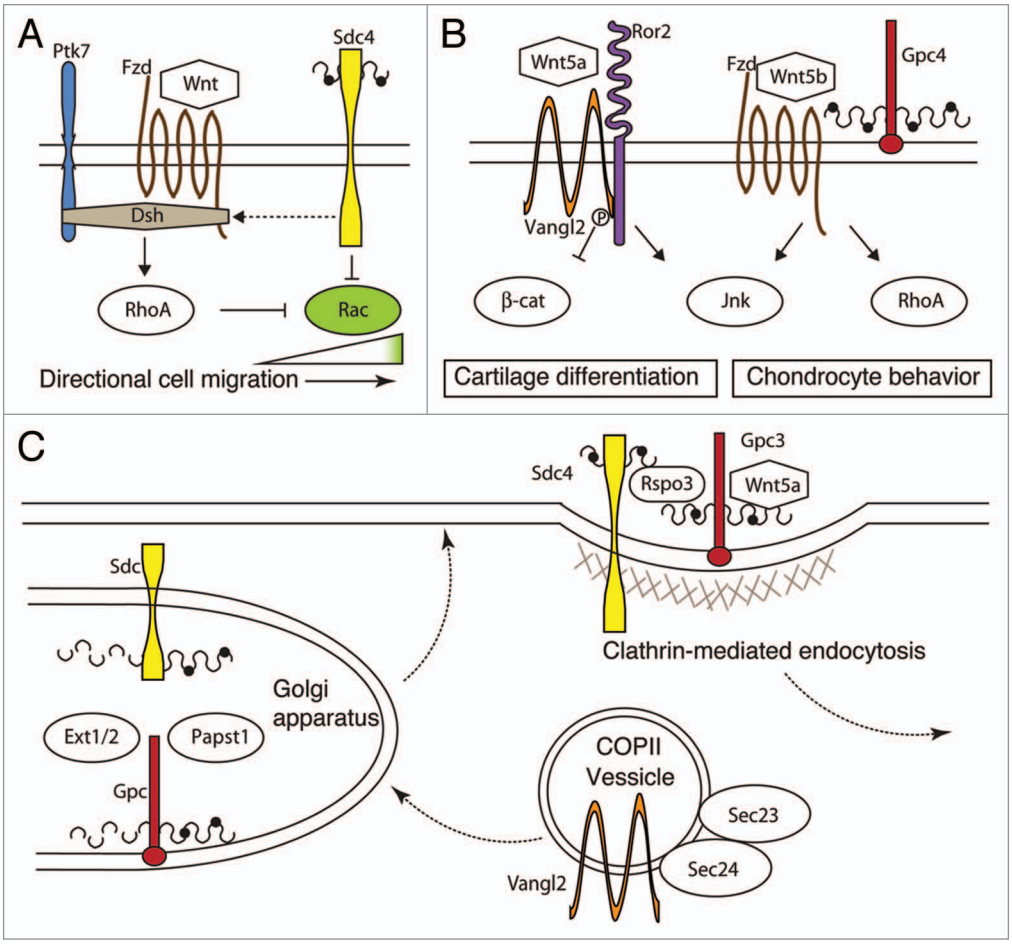

Figure 1. The role of PCP signaling in craniofacial development. The core planar cell polarity (PCP) signaling molecules, Frizzled (Fzd), Disheveled (Dsh) and Van Gogh-like 2 (Vangl2), interact with multiple proteins to transmit planar cell polarity information to and from cells. (A) In migrating neural crest cells, Wnt/PCP signaling activates RhoA to inhibit Rac activity in the trailing edge of the cell. Both Syndecan 4 (Sdc4) and protein tyrosine kinase 7 (Ptk7) can interact with Dsh, while Sdc4 can directly inhibit Rac. (B) During craniofacial cartilage formation, core PCP proteins' interaction with Ror2 and proteoglycans, such as Glypican 4 (Gpc4), inhibit Wnt/β-catenin signaling and activate RhoA and Jun signaling. (C) Transport of the proteins involved in PCP processes by a Sec23/24-dependent mechanism or their modification in the Golgi is essential for their function. A clathrin-mediated endocytosis process requiring R-Spondin 3 (Rspo3) and Wnt5a leads to the removal of Sdc4 and Gpc3 from the membrane. Perturbation of any of these processes affect Wnt/PCP signaling and craniofacial development.

modification take place. The Sec23 and Sec24 group of proteins are known to heterodimerize, where Sec24 proteins selectively bind cargo, and Sec23 proteins help to create the structural part of the COPII coat.[50,51] There are four known *sec24* genes in mammals, a through d. While there is some functional redundancy between *sec24a* and *b* and between *sec24c* and *d*, each has been shown to bind preferentially to specific protein cargo.[52] Interestingly, *sec24b* and *sec24d* are crucial for different aspects of PCP signaling. Two groups independently identified mouse lines with mutations in *Sec24b* with classical Wnt/PCP defects, such as neural tube closure defects known as craniorachischisis, abnormal organ of Corti hair cell arrangement and cardiac defects.[53,54] Disruption of *Sec24b* results in abnormal trafficking of VANGL2, a key modulator of PCP cellular morphogenesis, but not other membrane-bound proteins. In zebrafish, *sec23a* and *sec24d* mutants have smaller disorganized craniofacial cartilage elements composed of rounded cells that are unable to secrete Col2a1a and other extracellular matrix (ECM) proteins that are stuck in the rough ER.[47,49] The malformations of craniofacial cartilage elements of the *sec24d* mutant are not a result of a reduction of cell number but are due to abnormal cell shape, resembling phenotypes of the *wnt5b* and *gpc4* mutants.[13,21,49] While the authors of these papers suggest that the cell shape defect is due to the loss of ECM, they do not preclude the possibility that Wnts and other signaling molecules could be affected by the loss of *sec24d*. Not surprisingly, studies in humans have found that cranio-lenticula-sutural dysplasia (CLSD), a disorder in which the anterior fontanels are developmentally delayed, results from a mutation in the *SEC23A* gene.[55] Patients with CLSD also present with prominent foreheads, hypertelorism, prominent brow ridges and broad noses. Based on the studies reviewed here, proteoglycans, such as *gpc4* and *sdc4*, and their required posttranslational modifications and transport play a critical role in the PCP of craniofacial skeletal formation.

Palate Formation

Several developmentally important signaling pathways control the complex formation of the secondary palate.[56] Both the β-catenin-dependent and -independent Wnt signaling pathways have been implicated in this process. Deficiency of two Wnt ligands associated with the β-catenin-dependent pathway, the *Wnt9b*$^{-/-}$ mouse knockout and humans with mutations in *WNT3,* result in clefting of the palate.[57,58] The β-catenin-independent signaling molecule Wnt11 was also proposed to be important in the final steps of secondary palate formation.[59] In addition, WNT5A deficiency leads to a complete cleft formation in mice.[60] WNT5A controls directional cell migration and proliferation in this process and is mediated by Ror2. Frizzleds, the typical Wnt receptors, also play a role in palate closure; in particular, mouse embryos deficient in *Fzd2* frequently develop cleft palate. This defect is fully penetrant in double *Fzd1*$^{-/-}$*; Fzd2*$^{-/-}$ mutants.[61] In addition, *Fzd1*$^{-/-}$*; Fzd2*$^{-/-}$ mice exhibit shortened lower jaws (hypognathia). Interestingly, WNT9A and WNT3 were shown to induce robust induction of β-catenin signaling by reporter expression when co-expressed with FZD1 or FZD2 in contrast to WNT5A or WNT11, which did not elucidate such a response.[61] While a strong interaction was observed between the core PCP genes *Vangl2* and *Fzd1* and *Fzd2* in neural tube closure, no enhancement of palate formation defects has been demonstrated.[61] In addition, *Vangl2*$^{-/-}$ mouse mutants do not have defects in palate closure,[62-64] leaving the question of the role of PCP signaling in palategenesis open.

Conclusion

Much of what is known about the role of the Wnt/PCP signaling pathway in craniofacial development is projected from its role in other tissues. While manipulation of the individual members of the pathway clearly leads to a disruption of normal craniofacial development, in most cases, the targets of the pathway have yet to be identified. Therefore, in order to gain a clear understanding of the pathway's role within craniofacial development, it needs to be determined whether the downstream targets of PCP signaling within other tissues also play a role within craniofacial development.

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