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Review

New directions in craniofacial morphogenesis

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ABSTRACT

The vertebrate head is an extremely complicated structure: development of the head requires tissue–tissue interactions between derivates of all the germ layers and coordinated morphogenetic movements in three dimensions. In this review, we highlight a number of recent embryological studies, using chicken, frog, zebrafish and mouse, which have identified crucial signaling centers in the embryonic face. These studies demonstrate how small variations in growth factor signaling can lead to a diversity of phenotypic outcomes. We also discuss novel genetic studies, in human, mouse and zebrafish, which describe cell biological mechanisms fundamental to the growth and morphogenesis of the craniofacial skeleton. Together, these findings underscore the complex interactions leading to species-specific morphology. These and future studies will improve our understanding of the genetic and environmental influences underlying human craniofacial anomalies.

Introduction

The tissues of the vertebrate head are derived from all the germ layers: ectoderm, mesoderm and endoderm together with the "fourth" tissue layer, the neural crest. The ectoderm forms the epidermis, nervous system and components of the sense organs such as the lens. The endoderm lines the pharynx and contributes to a number of specialized glands. During embryogenesis, both the ectoderm and endoderm signal to the intervening facial mesenchyme. This mesenchyme is composed of mesoderm and neural crest. The mesodermal component later gives rise to the voluntary muscles and endothelial cells while the neural crest-derived mesenchyme forms the majority of the craniofacial skeleton. Given the significant contribution of the neural crest to the craniofacial structures, craniofacial anomalies can often be traced to changes in the neural crest.

In this review, we focus on later events in the morphogenesis of the face, including contributions from the surrounding ectoderm and underlying endoderm. Several recent studies have highlighted specialized signaling centers which control the outgrowth and polarity of the facial structures. These critical anatomical regions have been identified by a combination of experimental approaches, including analysis of embryonic inductive capabilities, molecular signatures and genetic requirements. Frequently, these anatomical structures express molecules, such as BMPs, FGFs and Wnts, which play multiple roles in facial morphogenesis: first, in specification and growth of tissues and later, during differentiation and skeletogenesis.

We will first review the general development of the head and describe the signaling centers that pattern each region, particularly recent data describing instructive cues from the anterior endoderm and the frontonasal ectodermal zone (FEZ), which control the initial outgrowth of the upper face. Finally, we will discuss new data linking molecular cues to cell movements and cell polarity in craniofacial development.

Development of the face and head

In general, development of the craniofacial structures can be considered in five discrete stages subsequent to the patterning of the germ layers. First, the neural crest is induced at the ectoderm/neuroectoderm border. This is followed by movement of the cranial neural crest into the presumptive facial primordia (Figs. 1A, B; (Johnston, 1966; Le Lievre, 1978; Le Lievre and Le Douarin, 1975; Sadaghiani and Thiebaud, 1987) and reviewed in (Creuzet et al., 2005)). Subsequently, regional proliferation of the neural crest leads to the formation of outgrowths called facial prominences (Figs. 1E, F and 3G, H). Next, the facial prominences fuse to presage the mature form of the face (Figs. 2A, B). Finally, the developing face is shaped by

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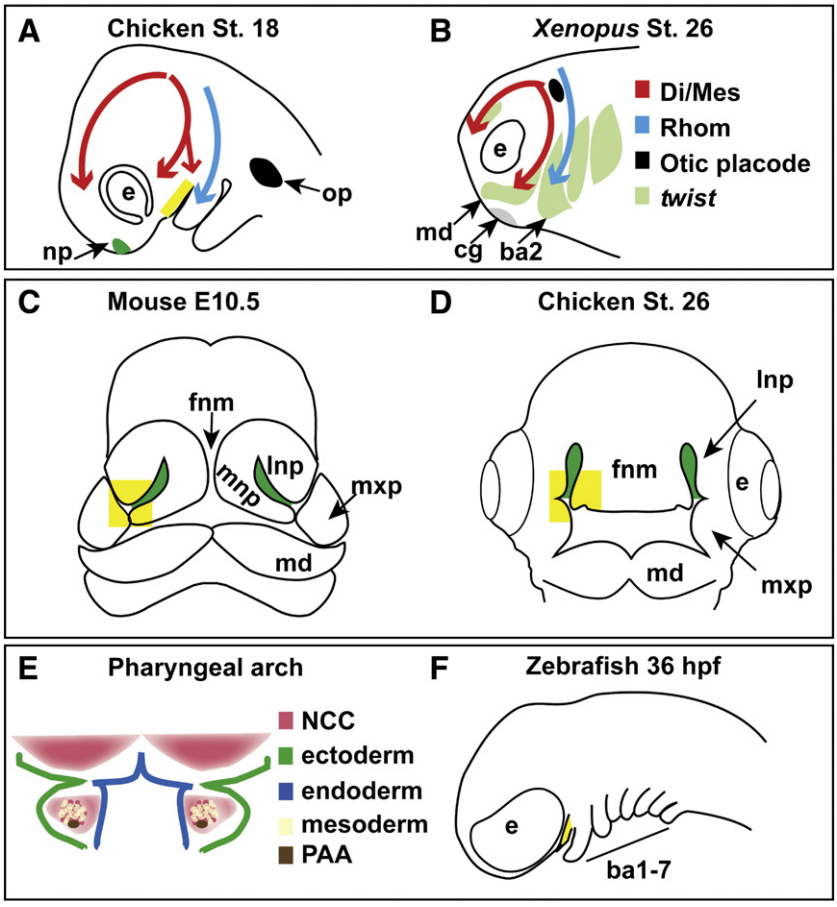

Fig. 1. Craniofacial anatomy. (A, B) Neural crest migration. (A) Lateral view of a stage 18 chicken embryo. Migration of the cephalic neural crest is depicted. Red: diencephalic and mesencephalic crest contributes to the upper face. Blue: rhombencephalic and posterior mesencephalic crest contribute to the mandibular arch. Maxillary prominence highlighted in yellow. (B) The pathways used by the migrating neural crest in the *Xenopus* embryo, superimposed over *twist* expression (green). *Twist* marks the final position of the cranial neural crest. Red: mesencephalic crest; Blue: hyoid crest from the rhombomeres. (C, D) Schematic frontal views of e10.5 mouse and stage 26 chicken embryos. (C) The mouse facial prominences (mnp, md, and mxp) surround the stomodeal opening. Note: the mnp are much more prominent in the mouse than in the chicken face. (D) The midline of the chicken face is much flatter than the mouse. Fusion of the lip will take place at the intersection of the tissues indicated in yellow. Green indicates nasal pits. (E) Frontal section through the first arch depicts tissue organization in the facial prominences. The surface ectoderm (green) encapsulates the neural crest derived mesenchyme (pink), which surrounds and later infiltrates the mesoderm (yellow). The pharyngeal arch arteries (brown) are in the core. The pharyngeal endoderm (blue) lines the pharynx. (F) Lateral view of a zebrafish embryo at 36 hpf, the maxillary prominences (yellow) and branchial arches are present. Key: ba, branchial arch; cg, cement gland; Di/Mes, mandibular stream, from the diencephalon/mesencephalon; e, eye; fnm, frontonasal mass; lnp, lateral nasal prominence; md, mandibular prominence; mnp, medial nasal prominence; mxp, maxillary prominence; NCC, neural crest cells; PAA, pharyngeal arch artery. Rhom, hyoid stream, from rhombencephalon; Fig. 1D modified from Cruezet, 2005; 1E drawn after Sadaghiani and Thiebaud, 1987.

directional growth of the skeleton (Figs. 2E, H, 3F). As many excellent reviews have focused on steps one and two, the initial specification and migration of the neural crest (including (Knecht and Bronner-Fraser, 2002; Le Douarin and Kalcheim, 1999; Sauka-Spengler and Bronner-Fraser, 2008), we will focus this review on the later stages of facial development.

As the neural crest migrates into the face, the cranial placodes, which are specialized ectodermal thickenings, differentiate. Placodes, with some contributions from the neural crest, give rise to the cranial ganglia and components of the sensory organs such as the lens of the eye, and olfactory glia. Both the otic and olfactory placodes express many growth factors and may influence the formation of the face. For detailed reviews of placodal specification, development and differentiation the reader is referred to the following excellent and extensive reviews (Baker and Bronner-Fraser, 2001; Schlosser, 2006; Streit, 2004).

Development of the viscerocranium during mid-organogenesis

By e9.5 in mice, or week 4 of gestation in humans, the facial primordia consist of five swellings called facial prominences or processes (Figs. 1C, D). The midline of the upper face is composed of the frontonasal mass in the chicken, or the medial nasal prominences and frontonasal mass in mouse embryos. Compared with the mouse, the chicken has flatter and less distinct medial nasal prominences (Figs. 1C, D). The frontonasal mass and medial nasal processes will develop into the midface and contribute to the forehead, ridge of the nose and the primary palate, which includes the premaxillary segment of the upper jaw. The sides of the nose and cheeks develop from the lateral nasal and maxillary prominences respectively, while the mandibular processes form the lower jaw. These five prominences encircle the stomodeum, or primitive mouth.

During the early pharyngeal arch stages, vertebrate embryos have substantial morphological homology, although the number of arches has changed during evolution (Kuratani, 2005; Meulemans and Bronner-Fraser, 2002). The mandibular primordium, or lower jaw, develops from the first branchial arch (Figs. 1E, F). The branchial, or pharyngeal, arches are metameric structures consisting of an outer ectodermal layer and an inner endodermal layer. Sandwiched between these two layers are mesodermal cores which are initially surrounded by neural crest-derived mesenchyme (Fig. 1E; (Grenier et al., 2009; Kuratani, 2005; Meulemans and Bronner-Fraser, 2002; Noden, 1982; Schilling and Kimmel, 1994). Signals from the first branchial arch ectoderm are required for the osteogenic commitment of the ectomesenchyme (Hall, 1978; Tyler and Hall, 1977).

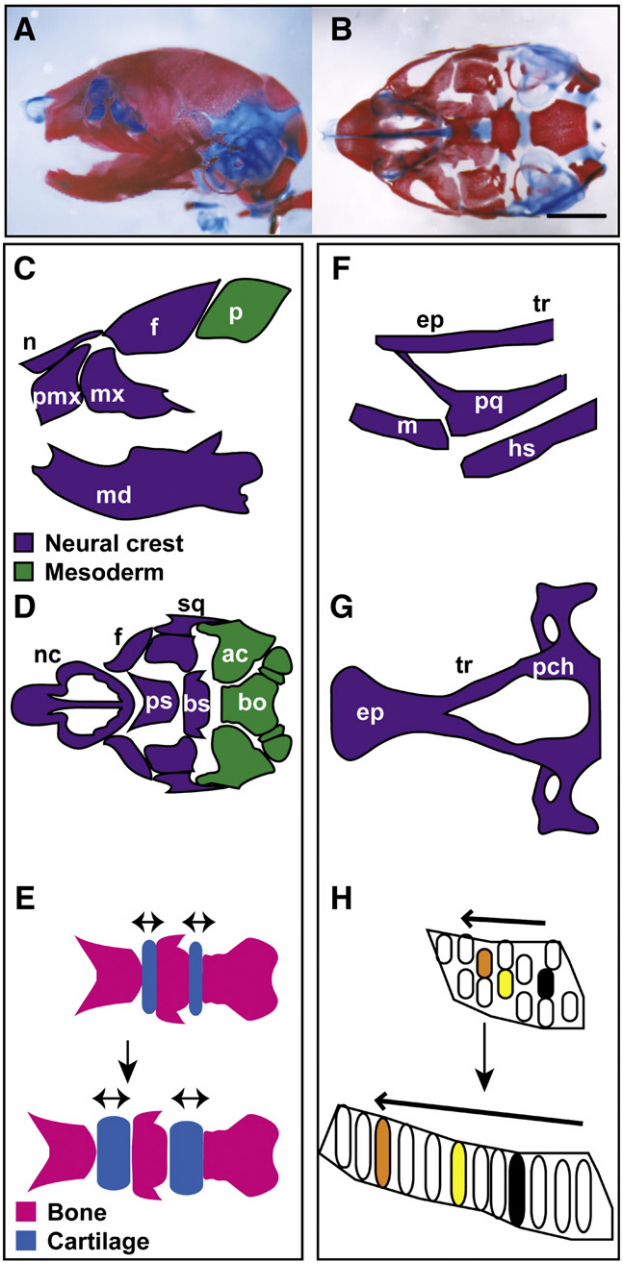

Fig. 2. Craniofacial skeleton. All drawings with distal to the left, lateral view is dorsal to top. (A, B) Mouse embryos (e17.5) stained for bone (alizarin red) and cartilage (alcian blue). (A) Lateral view. (B) View of the cranial base. Scale bar in B: 2 mm for both A and B. (C–E) Mouse anatomy: the skull of a late stage mouse embryo consists of neural crest (purple) and mesodermally (green) derived structures. These two tissue types abut in the skull between the frontal and parietal bones (C), and in the cranial base between the basioccipital and basisphenoid (D). (Modified from McBratney-Owen 2008.) (E) Directional growth of the cranial base. The cranial base consists of back-to-back growth plates. The joints, or synchondroses, are depicted in blue. These expand in the directions of the arrows, resulting the growth and extension in the upper face. (F–H) Zebrafish anatomy: the zebrafish head is made up of neural crest derived cartilages (purple). Osteogenesis begins after 4 days post-fertilization. (F) Lateral view of the upper and lower jaws at 2 days post-fertilization. (G) Dorsal view of the ethmoid cartilage, which provides support to the palate. (H) Outgrowth of the hyosymplectic cartilage. The cells initially are in small condensations. Through neighbor–neighbor rearrangements, possibly through PCP signaling, they align, giving directional outgrowth. KEY: ac, auditory capsule; bs, basisphenoid; bo, basioccipital; ep, ethmoid plate; f, frontal; hs, hyosymplectic; m, Meckel's cartilage; md, mandible; mx, maxillary bone; n, nasal; nc, nasal conchae; p, parietal; pch, parachordal; pmx, premaxillary bone; pq, palatoquadrate; ps, presphenoid; sq, squamosal; tr, trabecular cartilage.

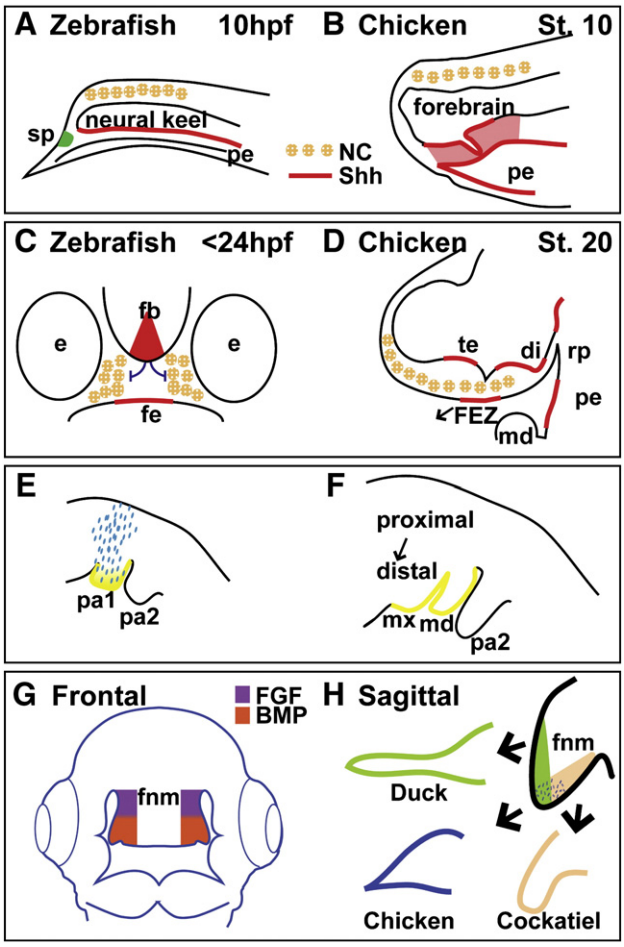

Fig. 3. Signaling at successive stages in craniofacial development. (A) Lateral section, zebrafish. In the zebrafish, at 10 hpf, Shh-expressing cells in the anterior neural keel (red) signal to the prospective stomodeal precursors (SP), rather than the distant neural crest (NC). This renders the facial ectoderm competent to induce the condensation of migrating neural crest. (B) Lateral section, chicken. In the chick embryo, at Stage 10, *Shh* (in red) is required within the pharyngeal endoderm (pe) and forebrain for jaw development. (C) Frontal section, zebrafish. Later, when the first arch neural crest migrates around and underneath the eye, *Shh* signals from the ventral forebrain (fb) preventing the cells from migrating into the midline. Subsequent to this schematic, at 24–30 hpf, the neural crest becomes adjacent to the facial ectoderm (fe); by these stages, the facial ectoderm is likely to be the main source of *Shh*. (D) Lateral section, chicken. At stage 20, the frontonasal ectodermal zone (fez) is evident. At this point, *Shh* (and *Fgf8*, not shown) from the FEZ signals to the adjacent neural crest to control outgrowth (in the direction of the arrow) of the facial prominences. Shh from the forebrain is thought to signal via the neural crest to induce the FEZ. (E–F) Patterning of pharyngeal arch 1. (E) The maxillomandibular crest (blue) expresses Ednra and migrates towards pharyngeal arch 1 (pa1). The ectoderm and endoderm in pa1 express endothelin 1 (yellow). (F) A few hours later, the maxillary and mandibular prominences become apparent. This is followed by a period of proximal-distal outgrowth shaping the jaws. (G, H) Regionalized proliferation in the avian frontonasal mass. (G) Medial–lateral proliferation regulates length of the beak. Schematic of stage 26 chicken face. In cranial regions mesenchymal cell proliferation (purple) is responsive to FGF signaling (Szabo-Rogers et al., 2007), while adjacent to the oral cavity, tissues marked in red are responsive to BMP signaling (Wu et al., 2006). Later, the growth zones merge medially in the chicken, while remaining lateral in the duck (not shown). (H) Proximal-distal proliferation regulates the size and angle of beak outgrowth. A sagittal section through the frontonasal mass (fnm) of an idealized stage 28 avian embryo shows regionalized proliferation domains. The broader, distally placed growth zone (green) contributes to the long horizontal beak of the duck. A more medial growth zone contributes to the intermediate size and angle of the chicken beak (blue stipple). The cockatiel has a proximal growth zone (beige), resulting in a shallow, downward-pointing beak. Figure modified from (Wu et al., 2006). Key: BMP: bone morphogenetic protein; di: diencephalon; e: eye; fb: forebrain; fe: facial ectoderm; FEZ: frontal ectodermal zone; FGF: fibroblast growth factor; fnm: frontonasal mass; md: mandibular prominence; mx: maxillary prominence; nc: neural crest; pa: pharyngeal arch; pe: pharyngeal endoderm; rp: Rathke's pouch; Shh: Sonic hedgehog; te: telencephalon; sp: stomodeal precursors.

The development of the upper face requires complex morphogenetic movements and occurs in two stages: an early phase for lip development, and a later phase for secondary palatogenesis. The upper lip or primary palate is formed in the mouse by e11.5 and before week 10 in human embryos. Together with the maxillary prominences, the lateral and medial nasal processes meet and fuse to form the upper lip (marked in yellow, Figs. 1C, D); however, precise fate mapping is still needed to determine relative contributions of the facial prominences to the primary palate. If fusion fails, cleft lip can occur, potentially affecting the development of the secondary palate. Subsequently, the medial nasal prominences merge along the midline and differentiate into the nasal septum, which is hypothesized to be a key structure that controls outgrowth of the midface.

Secondary palatogenesis is a complex and poorly understood process. It is dependent on the appropriate growth of both the mandibular and maxillary primordia; defects in either can result in cleft palate. The palatal shelves initially form as bilateral swellings of the maxillary prominence, extending along the lateral walls of the oropharynx. This stage is characterized by the coordinated proliferation and apoptosis of the undifferentiated ectomesenchyme. In this first stage, the palatal shelves grow vertically to flank the tongue. The second stage of palatogenesis relies on morphogenetic movements/growth of the lower jaw, to lower the tongue relative to the palatal shelves. Once the tongue has descended, the palatal shelves rotate towards the midline. The forces driving palatal shelf rotation are unclear but appear intrinsic to the shelves (Gritli-Linde, 2007). A distinct horizontal growth phase then follows, resulting in palatal shelves abutting at the midline. The palate fuses by e15.5 in the mouse, or roughly 12 weeks of gestation in humans. Finally, the fused palatal mesenchyme undergoes differentiation and ossification to form the mature structure dividing the nasal and oral cavities. Palatal morphogenesis is more thoroughly summarized in recent reviews (Gritli-Linde, 2007; Jiang et al., 2006).

Although palate development has typically been studied in the mouse, recent studies have suggested that the zebrafish embryo will be a useful system to investigate secondary palatogenesis, particularly for tracking cell migrations (Eberhart et al., 2008). Interestingly, directed cell migration has been observed in cultured mouse palatal shelves. However, it is not yet clear if this cell migration is required for morphogenesis of the palatal shelves (He et al., 2008).

Development of the visceral skeleton then shapes the facial structures (Fig. 2). For example, the maxillary and mandibular bones condense bilaterally, via intramembranous ossification (i.e. bone that forms without a cartilaginous precursor), in centers lateral to the nasal capsule (Kaufman and Bard, 1999; Tyler, 1978; Woo, 1949). Secondary cartilages then arise within the periosteum of the mandibular bone in response to force/movement, forming at the condylar head, mandibular angle and coronoid processes. The upper and lower jaws subsequently expand downward and forward through synchronized growth. How this growth is coordinated is still unclear (Depew and Compagnucci, 2008).

Development of the neurocranium during late organogenesis

The murine skull vault is of mixed origin, with neural crest condensations forming the frontal bone, and mesoderm contributing to the parietal bone (Fig. 2C (Jiang et al., 2002; Yoshida et al., 2008)). In contrast to the mouse, the avian frontal bone has a dual origin, with the anterior and posterior parts being formed by neural crest and mesoderm respectively (Couly et al., 1993; Noden, 1986). The skeletal structures of the cranial vault first appear bilaterally as intramembranous condensations that expand medially (Quarto and Longaker, 2005; Rice et al., 2000; Slater et al., 2009; Trueb and Hanken, 1992). The developing bones subsequently meet at sutures, which are regions of undifferentiated mesenchyme. These sutures, or joints, contribute to the growth of the developing bones. Both the interfrontal and coronal sutures contain neural crest derived mesenchyme (Jiang et al., 2002). In humans, these sutures remain open throughout early childhood, accommodating the expansion of the brain. Premature fusion of these sutures, or craniosynostosis, results in brain malformations, and the distortion of the skull, as the brain cannot grow appropriately (Rice, 2008). In the mouse, the posterior frontal sutures fuse by postnatal (p) day 45, while the remaining sutures remain patent (Bradley et al., 1996). Development of this region of the head is intimately linked with the brain and surrounding membranes, such as the *dura mater*. As the brain develops it forces growth of the bones at the sutures. Tensions at the sutures, combined with signals from the *dura mater*, are required to maintain suture patency (Opperman et al., 1993). The direct relationship between cranial vault morphogenesis and the growth of the brain is clearly illustrated by microcephaly and macrocephaly, where the skull is smaller or larger respectively (Van Den Bosch, 1959).

The cranial base, or basicranium, protects and supports the brain. However, because of its location and anatomical connection with the face, morphogenesis of the cranial base is also crucial for correct shaping of the facial morphology (Figs. 2D, E). In contrast to the cranial vault, the cranial base undergoes endochondral ossification, using a cartilaginous framework/scaffold to shape the bones, as in the axial and appendicular bones. Thus, achondroplasia patients, who have defects in chondrogenesis, have largely normal craniofacial skeletons, except for a shortened cranial base (Chen et al., 1999; Matsushita et al., 2009). The cranial base synchondrosis, or cartilaginous joint, resembles the epiphyseal growth plate of the long bones; with the exception that growth is bidirectional. This configuration of "back-to-back" growth plates is unique to the cranial base (Fig. 2E). Cranial base anomalies have also been identified in human malformations that involve cleft palate and craniosynostosis, as well as in Down syndrome (Michejda and Menolascino, 1975; Richtsmeier and Deleon, 2009).

Several recent studies have explored the origins of the mouse and chick cranial base. The anterior cranial base is largely derived from the neural crest, whilst the posterior cranial base is formed by the paraxial mesoderm (Couly et al., 1993; Le Douarin et al., 1993; McBratney-Owen et al., 2008). A number of groups have also examined late embryonic and postnatal ossification of the cranial base, with a particular focus on the synchondroses that serve as the major growth centers (Ingervall and Thilander, 1972; Koyama et al., 2007; Roberts and Blackwood, 1983; Young et al., 2006). Finally, the anterior and posterior portions of the cranial base ossify at different rates, with the anterior region ossifying much later (Nie, 2005). It is unclear whether these differences in the ossification rate are due to the different tissue origins of the cranial base.

Patterning the craniofacial complex: Novel signaling centers

Neural crest and facial morphology

Patterning of the branchial arches is complex and requires cranial neural crest signaling, reciprocal signals from the arch mesoderm, as well as contributions from the underlying pharyngeal pouch endoderm and overlying ectoderm (Fig. 1E). The neural crest cells that populate the branchial arches move in stereotypical streams from the neural tube (Figs. 1A, B). An unresolved question in the field is centered on the patterning that is inherent to the migrating neural crest cells. Some studies suggest that the neural crest cells are intrinsically pre-patterned to form the correct skeletal morphology and dictate the program for development of the surrounding tissues (Schneider and Helms, 2003; Tucker et al., 1999). For example, transplanting pre-migratory neural crest cells, from quail into duck embryos or *vice versa*, results in a facial morphology that resembles

the species of the neural crest *donor* and not that of the host. The timing of development of the skeletal structures, together with the timing and patterning of adjacent tissues, such as the ectoderm, are also determined by the donor neural crest (Eames and Schneider, 2008; Noden, 1983, 1986; Schneider and Helms, 2003; Tucker and Lumsden, 2004).

However, developmental pattern can be established by reiterative epithelial-to-mesenchymal signaling. These interactions occur while the neural crest cells are migrating and after they have arrived at their destination (Couly et al., 2002; Lee et al., 2001; McGonnell and Graham, 2002; Trainor et al., 2002; Trumpp et al., 1999). Some of these patterning centers appear to be established independently of the neural crest, likely from ectodermal or endodermal signals (discussed below). This would be consistent with the original grafting experiments of Noden which showed that the fate of ectopically transplanted neural crest is determined by environmental signals in the developing facial primordia (Noden, 1983).

Finally, within the caudal branchial arches, anterior–posterior identity appears to be determined by combinatorial Hox gene expression (Baltzinger et al., 2005; Hunt et al., 1995; Hunt et al., 1991; Minoux et al., 2009). However, the first arch neural crest does not express any Hox genes and appears to be the ground pattern. Here, regionalized expression of *Distal-less (Dlx)* homologues, which act downstream of Edn signaling, is thought to determine mandibular versus maxillary identity.

Endodermal signals patterning the facial primordia

The endoderm has two distinct roles in patterning the head: signals from the endoderm regulate cell survival and pattern the neural crest. In zebrafish, a lesion in Sox32 (*casanova*) results in the absence of the pharyngeal endoderm. As a result, these mutants do not develop the viscerocranium (mandible), as the neural crest cells are lost through apoptosis. In contrast, the neurocranium, which develops independently of the endoderm, is relatively unaffected (David et al., 2002). Through a series of elegant transplant experiments, David and colleagues (2002) found that the *casanova* neural crest could differentiate into chondrocytes when placed in a wildtype first arch. This suggests that the defects are not intrinsic to the neural crest. This was confirmed in the converse experiment, where wild-type endoderm grafted into mutants embryos, rescued chondrogenesis. Thus, the post-migratory mandibular crest requires cell survival signals from the endoderm (possibly FGF3) (David et al., 2002).

Similarly, in early chicken embryos, surgical ablation of the endoderm abolishes the development of the facial structures (Fig. 3B and (Couly et al., 2002)). By removing strips of endoderm before neural crest migration, Couly et al. (2002) showed that the most rostral endoderm was required for nasal cartilage morphogenesis, while the more caudal endoderm is required for the development of Meckel's cartilage and the mandibular joint. This loss of structures could be due to apoptosis, as described above in zebrafish. However, supporting a role for the endoderm in patterning, grafts of endoderm induced the formation of ectopic structures in host embryos, corresponding to the original rostral-caudal location of the donor endoderm (Couly et al., 2002). Rostral endoderm induced nasal cartilages while more caudal endoderm induced the formation of ectopic Meckel's cartilages. Furthermore, the orientation of these structures depended on the grafted endoderm: rotation of the endoderm by 180 degrees led to a misoriented Meckel's cartilage (Couly et al., 2002). This clearly shows that endodermal signals can pattern the facial primordia.

Shh may be the key endodermal signal. In the chicken, the loss of foregut endoderm can be rescued by application of Shh-soaked beads (Brito et al., 2006), and as grafting Shh-expressing cell pellets into the first branchial arch induced *Fgf8* and *Bmp4* expression, as well as supernumerary mandibular skeletal structures (Brito et al., 2008). Placing Shh cell pellets into the second branchial arch, or anterior to the first branchial arch, did not induce ectopic bone and cartilage development, suggesting that the response to Shh depends on the identity of the responding tissue, or on the Shh signal being received at the correct time in development. An alternative explanation is that Shh is required for cell survival of neural crest cells throughout the facial primordia but has an additional distinct role in the first arch, where Shh signaling is also required for patterning. All of these possibilities need to be explored.

Patterning of the maxillary versus the mandibular primordia

Although the mandibular and maxillary primordia are derived from similar populations of neural crest cells, and share many molecular characteristics, they develop into distinct skeletal structures. This may reflect differences within the neural crest populations, within the pharyngeal arch, or in the post-migratory environment. Hh can elicit different responses from the dorsal and ventral portions of the pharyngeal arch, which are fated to become the maxillary and mandibular primordia, suggesting regionalization prior to the arrival of the neural crest. In the mandibular primordia, viral overexpression of *Shh* induces the expression of *Fgf8* within the over-expressing cells (Haworth et al., 2007). In contrast, in the maxillary prominence, ectopic *Shh* upregulates *Fgf8* expression only in a population of epithelial cells adjacent to the *Shh* expressing cells (Haworth et al., 2007). This differing sensitivity suggests that there is very early (before the arrival of the neural crest) regionalization of the dorsal and ventral portions of the pharyngeal arch, which are fated to become the maxillary and mandibular primordia, respectively.

Recent data suggests that endothelin signaling may set apart the mandibular portion of the first pharyngeal arch. The ligand, endothelin1 (Edn1/ET1) is expressed throughout the endoderm and mesoderm of the mandibular arch while its receptor, Ednra, is expressed in the neural crest (Figs. 3E, F) (Clouthier et al., 2003; Ozeki et al., 2004; Sato et al., 2008). Mice lacking *Edn1* or *Ednra* exhibit a homeotic transformation of the lower jaw into maxillary structures. Conversely, overexpression of Edn1 is sufficient to induce mandibular fates from maxillary precursors (Clouthier et al., 1998; Sato et al., 2008). Blocking Ednra function in chicken and rat embryos also leads to mandibular arch defects, suggesting evolutionary conservation of Edn1 activity in the face (Kempf et al., 1998; Kimmel et al., 2003; Spence et al., 1999). These studies did not evaluate whether a transformation of skeletal structures occurred, although injecting Edn1 protein into zebrafish branchial arches does cause a dorsal-to-ventral transformation of Meckel's cartilage supporting a role of Edn1 in jaw patterning (Kimmel et al., 2003; Kimmel et al., 2007).

Genetic inactivation of *Ednra* in mouse and zebrafish, or pharmacological antagonism of Edn function, has shown that Edn is not required for the migration of the cephalic neural crest. In fact, chicken embryos do not express *Ednra* in the migratory crest cells (Clouthier et al., 1998; Kempf et al., 1998; Kimmel et al., 2001). In addition, the lower jaw defects of zebrafish *edn1* mutants can be rescued by application of exogenous Edn1 protein after neural crest migration (Miller et al., 2000).

Taken together, these data strongly suggest that Edn signaling is required in the post-migratory cranial crest to pattern the vertebrate jaw. In the mouse, *Edn* is required between e8.5 and e9.5 suggesting that maxillary versus mandibular identity is established during this developmental window just prior to, and as the neural crest are populating the primordia (Fukuhara et al., 2004; Ruest and Clouthier, 2009). This time point is consistent with that identified by Haworth and colleagues in their *Shh* overexpression studies: both suggest very early regionalization of the maxillary and mandibular primordia (Haworth et al., 2007). Oddly, in *Xenopus,* Edn1 signaling is also required for neural crest induction, survival and subsequent migration (Bonano et al., 2008; Meulemans and Bronner-Fraser, 2002).

Downstream targets of Endothelin include the *Dlx* transcription factors: mouse mutants lacking *Dlx5/6* recapitulate the craniofacial phenotype of Edn1/Endra mutants (Beverdam et al., 2002; Depew et al., 2002). The nested expression domains of these genes led to the proposal that a combinatorial code of *Dlx* genes patterns the jaw primordia (Depew et al., 2005). Interactions with other signals, such as Fgf8, may also be important (Tucker et al., 1999; Fukuhara et al., 2004). In addition, Edn signaling interacts genetically with the transcription factor *Mef2C* in fish and mouse (Miller et al., 2007; Verzi et al., 2007) Analysis of the mouse *Dlx5* and *Dlx6* enhancers suggests that MEF2C acts as a direct transcriptional activator of both these genes (Verzi et al., 2007). The homologous zebrafish gene, *mef2ca,* acts similarly, as *dlx5a/6a* expression is downregulated in *mef2ca* mutants, as are the Edn1 targets *gsc* and *bapx1* (Miller et al., 2007). These groups propose that graded levels of Edn1 signalling activity are interpreted by MEF2C to control differential *Dlx* expression; this needs to be further explored.

Patterning and outgrowth of the upper face: multiple sources of Hedgehog

Signals from the adjacent foregut endoderm control the development of the upper face (including the premaxilla and the nasal capsule). Here, these act together with additional signals from the neuroectoderm and facial ectoderm. The most anterior pharyngeal endoderm is necessary for the induction of the mesethmoid, a component of the nasal capsule, in chicken embryos (Benouaiche et al., 2008; Brito et al., 2006; Couly et al., 2002). Removal of this endoderm results in the loss of the mesethmoid. Consistent with this, grafting this portion of the endoderm into a new host can induce the formation of an ectopic nasal cartilage in Hox-negative neural crest, including within the presumptive lower jaw (Benouaiche et al., 2008). In each case, *Hh* signaling from the endoderm is required.

Likewise, Hh signaling is required for the formation of the anterior neurocranium (ANC; ethmoid plate and trabeculae) in zebrafish (Figs. 2F, G). In *smoothened* (*smo-)* mutants, which cannot transduce a Hh signal, the anterior neurocranium is absent. Normally, the skeletal precursors of the ANC condense on top of the stomodeal ectoderm (roof of the stomodeum), but in *smo-* mutants, the neural crest cells do not condense. Instead, they eventually migrate to an ectopic location posterior to the eye (Figs. 3A, C; (Eberhart et al., 2006)). As shown by mosaic analyses, this defect is due to loss of a signal within the ectoderm (that is, extrinsic to the neural crest). *Smo-* mutant neural crest can condense on wildtype stomodeal ectoderm. Wildtype neural crest cannot condense on the mutant stomodeal roof (Eberhart et al., 2006). Treatment of wildtype embryos with the Hh antagonist, cyclopamine, shows that Hh signalling is required at the end of gastrulation. These studies suggest that Hh protein from the ventral midbrain is required within the stomodeal precursors, before the neural crest migrates (Figs. 3A, C; (Eberhart et al., 2006)). This Hh signal probably induces an unknown factor in the stomodeal ectoderm which is required to direct condensation of neural crest cells in the appropriate location. The ability of ectodermal signals to induce the formation of skeletal structures was previously proposed by Thorogood in his “Flypaper” model (Thorogood, 1988). He proposed that local signals within the ectoderm (possibly Type II collagen) promote the formation of skeletal structures surrounding the developing brain and sensory organs. The above study nicely supports his model, although the candidate “Inducer/Flytrap molecule” still remains elusive.

Patterning and outgrowth of the upper face: Frontonasal Ectodermal Zone

Several recent studies describe regionalized signaling centers and proliferative zones in the facial primordia. Hu et al. (2003) have identified a region of the superficial facial ectoderm, the frontonasal ectodermal zone (FEZ), which is defined by the juxtaposition of *Fgf8* and *Shh*-expressing domains. The FEZ is established just prior to the outgrowth of the frontonasal prominence and regulates the three-dimensional expansion and orientation of the upper jaw (Fig. 3B). Transplantation of an ectopic FEZ into the frontonasal mass or mandibular primordium of a host embryo. This study has shown that the FEZ is sufficient to induce growth of the underlying tissue (Hu et al., 2003). The FEZ does not repattern the mesenchyme, as the tissues retain their identity (as frontonasal mass versus mandibular primordia) but does determine the dorsal-ventral orientation of the ectopic beak (Hu et al., 2003). The signals from the FEZ include Fgf8 and Shh; however, these factors are not sufficient to substitute for the epithelial grafts, suggesting that other factors, possibly BMPs, are important (Abzhanov and Tabin, 2004; Hu and Marcucio, 2009; Hu et al., 2003; Wu et al., 2004).

The FEZ, *per se*, has not been identified in zebrafish embryos, but there is an apparent lack of outgrowth of the anterior neurocranium in *syu* (*sonic you*, *Shh*) or *ace* (*acerebellar*, *Fgf8*) mutants (Albertson and Yelick, 2005; Wada et al., 2005). In mouse and human embryos, the FEZ is found at the tips of each medial nasal prominence, while in the avian embryo the FEZ spans the entire frontonasal process. It has been proposed that variation in relative size and position of the FEZ determines species-specific shape and outgrowth of the upper face (Hu and Marcucio, 2009). Positioning the FEZ requires Shh expression from the forebrain (Figs. 3B, D; (Marcucio et al., 2005)). BMP signaling is also required, as inhibition of BMP signaling, by overexpression of the BMP inhibitor Noggin, prevents the formation of the FEZ (Foppiano et al., 2007).

The placodes are an additional source of inductive signals. Recent data suggest that the olfactory placodes are also a major source of FGF signaling, and are required for development of the lateral nasal prominences (Szabo-Rogers et al., 2009). Likewise, the otic placode expresses many growth factors, which will likely influence craniofacial development (Liu et al., 2002).

Species-specific outgrowth of the face

During pharyngula stages, the facial primordia are similar between species. Subsequent variation in signaling centers such as the FEZ may create species diversity by changing the patterns of proliferation and outgrowth in the face. These variations are nicely illustrated in several studies of avian embryos.

In the chicken, the frontonasal mass initially has bilateral regions of proliferation. As the embryo develops these two proliferation domains merge to form a single proliferation zone in the midline (Fig. 3G). Therefore, maximal growth occurs at the center, resulting in a narrow pointed beak. The duck, which has a broader beak, also has two lateral regions of proliferation. But, in contrast to the chick, the zones do not fuse and are maintained bilaterally (Wu et al., 2006; Wu et al., 2004). Maximal growth then occurs across the entire frontonasal mass, resulting in a broader beak. Signaling in the maxillary prominences is also important, where inhibition of BMPs leads to the formation of a narrower beak (Wu et al., 2006). Thus, changing the topography of the growth zones gives rise to remarkably different beak morphologies.

Furthermore, in comparisons of duck, chicken and cockatiel, the curvature of the beak also correlates with the position of the FEZ and regions of high proliferation in the embryo (Fig. 3G). In the duck, which has a flat straight beak, this is located distally. In contrast, in the cockatiel, proliferation is highest proximally, and this results in a pronounced curvature of the beak (Fig. 3H). Finally, in the chicken, the “growth zone” is found midway along the proximo-distal axis of the developing beak resulting in a beak with a slight curvature (Fig. 3H and (Wu et al., 2006)).

A few signaling pathways have been linked to morphological differences in beak size and shape. In Darwin's finches, which have a range of beak widths and lengths, *Bmp4* expression is wider in the facial mesenchyme of ground-dwelling finches with the broadest beaks (Abzhanov et al., 2004). In wild-type chicken embryos BMP-4 is sufficient to induce an ectopic "growth zone", resulting in a widening of the upper beak; in contrast, ectopic Noggin dramatically reduces the size of the beak (Abzhanov et al., 2004; Wu et al., 2006). The shape changes were associated with increased cell proliferation in the BMP4-treated beaks. In a separate study, microarray analyses led to the discovery that Ca2+-dependent calmodulin signaling also underlies beak shape and size (Abzhanov et al., 2006). In this case, higher levels of calmodulin signaling were linked to the formation of the long and pointed beaks of cactus finches.

Patterns of Wnt/β-catenin (canonical Wnt signaling) responsiveness in the facial prominences may also control species-specific differences in facial outgrowth (Mani et al., 2009). Brugmann et al. (2007) have shown that active Wnt signaling correlates with the regions of highest cell proliferation. They hypothesized that this results in the mouse face growing bilaterally (at the lateral nasal and maxillary prominences) with a midline furrow. In contrast, there is a single medial stripe of active Wnt signaling in the chicken face, which may lead to continued outgrowth of the beak at the midline (Jiang et al., 2006). Additional evidence for Wnt regulation of growth comes from the A/WySn inbred strain of mice, which has a naturally occurring incidence of cleft lip. The causative mutation lies in the *Wnt9b* gene, which signals via the canonical Wnt pathway (Juriloff et al., 2006). Parsons and colleagues have shown that A/WySn embryos have smaller facial prominences when compared to the C57Bl/6 strain, which have negligible rates of cleft lip. This has lead to the hypothesis that the small size of the facial prominences contributes to the higher rate of cleft lip in A/WySn mice (Parsons et al., 2008).

Finally, FGF signaling also controls a subset of the proliferative zones in the frontonasal mass. In particular, the mesenchyme nearest the nasal pit is the most responsive to FGF (Fig. 3G and (Szabo-Rogers et al., 2008). Together these data show that several pathways control facial outgrowth by regulating regional proliferation.

Novel concepts in facial morphogenesis

Roles for planar cell polarity and ciliogenesis

Recent studies have suggested that planar cell polarity (PCP) genes, originally discovered in *Drosophila* for their role in orienting cell structures (reviewed in (Strutt, 2008)), may regulate cell polarity and tissue morphogenesis in vertebrate systems (Wallingford, 2006). In addition to positioning cellular structures such as the stereociliary bundles in the cochlea (Dabdoub et al., 2003; Montcouquiol et al., 2003; Wang et al., 2006; Wang et al., 2005), PCP signaling coordinates cellular behaviors, such as cell intercalation and tissue movements (Gong et al., 2004; Wallingford and Harland, 2002; Wallingford et al., 2000).

During craniofacial development, these cell behaviors have been observed in zebrafish and *Xenopus*. Extension and outgrowth of cranial cartilages has been characterized in the second branchial arch-derived hyosymplectic cartilage in zebrafish (Fig. 2H and (Kimmel et al., 1998)). These studies have revealed that nearest neighbor relationships change over time, and strongly argue for convergent extension-like cell behaviors, similar to those that occur during gastrulation and neurulation (Kimmel et al., 1998). Cell movements have not been investigated in the extension of Meckel's cartilage or nasal cartilages in amniotes, but we hypothesize that the same principles apply.

In vertebrates, Fz/PCP signaling is activated by a non-canonical Wnt ligand (e.g. Wnt5a, Wnt11) binding to a Frizzled receptor (e.g. Fz6, 7, 3) and activating Disheveled (Fig. 4D). Disheveled (Dvl) then regulates the localization of PCP effector proteins: Strabismus (Van Gogh), Diego (Diversin/Inversin) and prickle in the apical membrane. The apical localization of these proteins initiates a downstream signaling cascade of relatively unstudied effectors that translate PCP signals into cell movements/rearrangements.

When Wnt/PCP signaling is disrupted in zebrafish, the jaw cartilages are present but are shortened, supporting the hypothesis that PCP signaling controls cellular behavior rather than patterning (Hammerschmidt et al., 1996; Piotrowski et al., 1996; Solnica-Krezel et al., 1996). For example, zebrafish with mutations in *knypek/glypican 4*, a component of the PCP pathway, have defects of cell intercalation in Meckel's cartilage (LeClair et al., 2009; Topczewski et al., 2001).

In mice, characterization of PCP gene function in craniofacial development is currently hampered by the lack of availability of mutants and functional redundancy. The exceptions are compound mutants in *Dvl*, which have neural tube and heart defects, both of which can be attributed to changes in PCP signaling (Hamblet et al., 2002). The facial defects have not been well characterized, but include a shortened jaw and tongue malformations. More recently, we have shown that mutation of the PCP effector *fuzzy* also leads to craniofacial anomalies, including a shortened mandible, cleft palate and exencephaly (Gray et al., 2009). Exencephaly is often linked to a failure of convergent-extension resulting in an open neural tube. The shortened mandible may be due to a failure of Meckel's cartilage extension, analogous to branchial arch cartilage extension in zebrafish. These combined phenotypes suggest new roles for planar cell polarity signaling in mammalian craniofacial development. Indeed, we have observed defects in the development of Meckel's cartilage, but a full morphological analysis has yet to be carried out. This new role for PCP signaling in the craniofacial skeleton would also be consistent with a recent report demonstrating polarized cells and cell reorientation during endochondral cartilage development of the long bones (Ahrens et al., 2009). Furthermore, analysis of mouse mutants in *Wnt5a*, which functions in the planar cell polarity pathway, showed that *Wnt5a* regulates directional cell migration in the palate (He et al., 2008). Taken together, these data give new insight into morphogenesis, and raise the possibility that the later shaping of the mature face may be directed by the PCP control of cartilage outgrowth in the mandibular primordia, and by extension, chondrogenesis in the cranial base and nasal cartilages.

In addition to the roles for PCP signaling in cell polarity and tissue movements, recent studies have linked PCP genes to ciliogenesis, which could shed new light on ciliopathies implicated in craniofacial syndromes (Fig. 4). Several proteins involved in PCP signaling localize to the base of cilia (Das et al., 2004; Jenny et al., 2005; Ross et al., 2005), including Dvl (Park et al., 2008). It is unclear whether loss of PCP signaling would affect cilia function *per se* but defects in cilia function/structure are likely to affect PCP signaling.

The primary cilium is a conserved organelle present on the surface of most vertebrate cells. Primary cilia are non-motile microtubule-based axonemal structures that play crucial roles as mechanical and chemical sensors. Because there is no protein synthesis in the cilium, assembly and maintenance of the cilium requires intraflagellar transport (IFT) (Fig. 4A). Thus, defective IFT proteins lead to defects in the structure or function of the primary cilium. These mutations have been associated with congenital human anomalies, including *situs inversus*, polycystic kidney disease and a variety of skeletal defects (reviewed in (Eggenschwiler and Anderson, 2007; Haycraft and Serra, 2008)).

Several rare pleiotropic diseases affecting the craniofacial skeleton also implicate the cilia. Most notable, oral facial-digital syndrome (OFD) and Bardet–Biedl syndrome (BBS) display ciliary dysfunction. Symptoms include stereotypical facial changes, as well as cleft palate and micrognathia (Ferrante et al., 2006; Tobin et al., 2008). Mutation

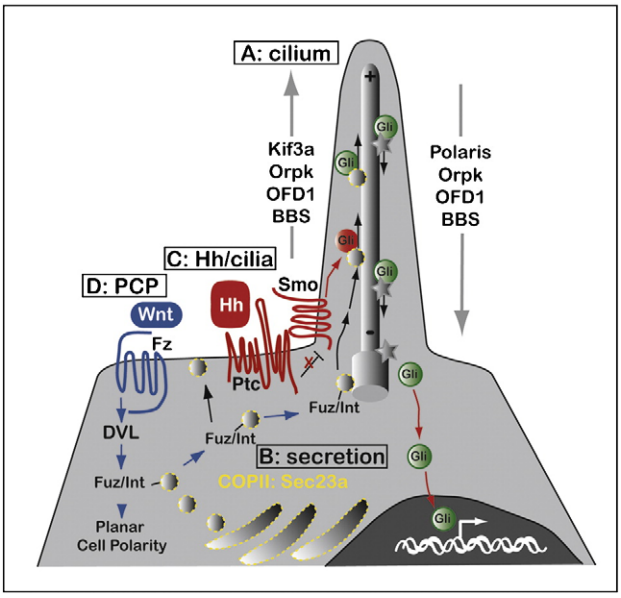

Fig. 4. The intersection of planar cell polarity, secretion and Hh signaling at cilia. (A) Simplified schematic of cilia. Because there is no protein synthesis in cilia, transport of cargo in the anterograde (towards the plus end) or retrograde (to the minus end) direction is critical for their function. Pictured is a subset of proteins implicated in ciliary transport and craniofacial morphogenesis. (B) Secretion has also been linked to craniofacial anomalies. Specifically, Sec23, a member of the COPII complex, which is critical for protein trafficking to the Golgi, causes cranio-lenticulo-sutural dysplasia. (C) Hedgehog signal transduction requires functional cilia. In the absence of ligand, transmembrane Patched inhibits the receptor Smoothened (Smo). Gli is processed to a repressor form (red). Binding of Hh ligand relieves this inhibition and allows the relocalization of the Smo receptor to the cilia, where Smo then recruits a complex that promotes Gli activator formation (green). This process appears to be dependent on Kif3a. (D) Planar polarity signaling may regulate secretion and ciliogenesis. During planar cell polarity signaling, non-canonical Wnts bind to the Frizzled receptor, activating disheveled (DVL). DVL then genetically regulates PCP effectors, including Fuzzy (Fuz) and Inturned (Int) as well as apical localization of other factors (not shown). Dvl, Fuz and Int have recently been shown to be required for ciliogenesis; this may involve regulation of the secretory pathway as Fuz has been shown to be required for the transport of proteins to the ciliary basal body.

of X-linked *OFD1* in mice has severe consequences, including a complete lack of cilia, early lethality in males and cleft palate in heterozygous female animals (Ferrante et al., 2006). Knockdown of *Ofd1* in zebrafish using morpholino oligonucleotides results in a short and stubby Meckel's cartilage composed of disorderly stacks of chondrocytes (Ferrante et al., 2009). Some of these defects may be linked to defective PCP signaling due to changes in the cilia.

Furthermore, Bardet–Biedl syndrome, which is characterized, in part, by craniofacial abnormalities, is due to a mutation in one of several of the BBS genes (*BBS1-14*); most of these genes localize to a protein complex in primary cilia. Analysis of *BBS6* mutant mice, which recapitulate the human condition, showed that the mice have a broader midface and hypoplasia of the nasal region (Tobin et al., 2008). Similar effects are seen in zebrafish BBS morphants. Knocking down *BBS8* in zebrafish resulted in severe hypoplasia of the branchial arches and mandibles. This resulted from defects in cranial neural crest cell migration, rather than proliferation or apoptotic effects (Tobin et al., 2008). While the cellular function of the BBS proteins is still unclear, the current hypothesis is that this complex is also required for ciliary vesicular transport (reviewed in (Jin and Nachury, 2009)). However, BBS4 mutant mice have defects in positioning of the hair cells in the ear, again implicating a relationship between cilia and PCP signaling (Ross et al., 2005).

The functions of the cilia are not just linked to planar cell polarity. Some phenotypes in ciliopathies appear to be due to perturbations in other signaling pathways, including Hedgehog, PDGF and Wnt (Corbit et al., 2008; Huangfu and Anderson, 2005; Schneider et al., 2005). In particular, the Hh receptor Smoothened is recruited to the primary cilia in a ligand-dependent manner, suggesting that HH signal transduction occurs via the cilia (Fig. 4A and (Corbit et al., 2005)). Indeed, in genetic models of primary cilia loss, key defects are linked to processing of the Gli transcription factors in response to Hedgehog signaling ((Haycraft et al., 2007; Huangfu and Anderson, 2005; Kolpakova-Hart et al., 2007) and reviewed in (Eggenschwiler and Anderson, 2007)). Similarly, *talpid*^{3} chicken mutants, which lack a centrosomal protein that is required for ciliogenesis, display aberrant Hh signal transduction. These mutants do not develop an oral cavity, have cyclopia and lack a frontonasal process, reminiscent of the craniofacial phenotype of the *Shh* null mouse mutants (Buxton et al., 2004; Davey et al., 2007; Yin et al., 2009).

Finally, recent studies have begun to explore the role of polarized growth and cell shape changes during skeletogenesis. For example, mouse mutants with a neural crest-specific disruption of *Kif3a*, an anterograde kinesin motor required for IFT and ciliogenesis, have shortened mandibles and defects in the development of the cranial base (Kolpakova-Hart et al., 2007). This raises the possibility that primary cilia are required to direct outgrowth of facial cartilages.

Mutation of a second HH ligand, *Indian hedgehog (Ihh),* results in craniofacial phenotypes attributed to failures in chondrogenesis and osteogenesis (Koyama et al., 2007; Maeda et al., 2007; Razzaque et al., 2005; St-Jacques et al., 1999). Recent comparison of development of the cranial base in the *Ihh* and *Kif3a* mouse mutants have found that the cranial bases are shortened anteroposteriorly, with abnormal synchondroses in both mutants. However, closer analysis revealed that the defects are complementary, as *Ihh* mutants display excessive hypertrophic chondrocytes and do not undergo endochondral ossification while the *Kif3a* mutant has the converse phenotype (Koyama et al., 2007). Furthermore, the *Kif3a*, but not the *Ihh*, deficient growth plates are disorganized, suggesting a role for primary cilia in chondrocyte rotation and orientation that is independent of *Hh* signaling. As a result, in the cranial base, the cilia may control both chondrocyte orientation and rate of ossification. These functions may be linked to PCP signaling (Koyama et al., 2007; Young et al., 2006).

These new studies, as well as identification of human ciliopathy genes, suggest important functions for cilia during embryonic development. However, surprisingly little is known about cilia during craniofacial development, especially in the patterning of the neural crest. In zebrafish, migrating neural crest cells are reported to have primary cilia (Tobin et al., 2008); however, it is unclear when these cilia become apparent or what signaling roles they might have within the neural crest.

Secretory pathway

An increasing number of human craniofacial anomalies, particularly those affecting chondrogenesis and osteogenesis, appear to stem from defects in the secretory pathway. The secretory pathway is required in all cells and ensures that extracellular proteins are appropriately transported through the endoplasmic reticulum and the Golgi apparatus to the cell surface. In chondrocytes and osteoblasts, the deposition of extracellular matrix proteins, such as collagen and matrix metalloproteases, is particularly susceptible to perturbations in the secretory machinery. The identification of mutations in *SEC23a* as causes of craniolenticulosutural dysplasia (CLSD) has identified a requirement for secretion in craniofacial morphogenesis. Sec23a is a component of the COPII complex, which coats vesicles and is required for protein trafficking to the membrane (Fig. 4B; (Boyadjiev et al., 2006; Fromme et al., 2007; Lang et al., 2006; Townley et al., 2008)). Mutation of zebrafish *sec23* (*crusher)* also leads to craniofacial defects. As a result of the human *SEC23* (F382L) mutation, collagen or proteoglycan secretion is impaired and tissues from patients show excessive protein accumulation in the endoplasmic reticulum, and presumably increased cell death from a “stress response” (Fromme

et al., 2007). In CLSD, the impaired secretory function in the osteoblasts of the calvaria results in open calvarial sutures, wide foreheads and high nasal bridges.

In epithelia, secretion is inherently polarized and a number of polarity complexes control protein sorting and distribution. These links have not been fully explored; however, the recent finding that the PCP effector *fuzzy* is essential for exocytosis in secretory cells, as well as being involved in ciliogenesis, highlights potential links between planar cell polarity, ciliogenesis and secretory vesicles (Gray, 2009; Kim et al., 2008). This link may not be that surprising as PCP components are also linked to apical-basal polarity in flies (Djiane et al., 2005). All together, these data suggest a link between Wnt/PCP signaling to cilia/Hh signaling and secretion (Fig. 4).

Conclusion

Over the past decade, studies of craniofacial development and morphogenesis have been significantly aided by the analysis of tissue specific mutants in mice, mutant screens in zebrafish and the availability of chemical tools (drugs, morpholino oligonucleotides, exogenous proteins) to perturb signaling. When combined with classical embryology techniques, these studies considerably extend our understanding of signaling interactions and molecular players that are essential for craniofacial morphogenesis. In parallel, the identification of new gene mutations in human anomalies has further pushed forward our understanding. However, when considering these studies, it has become increasingly clear that (with the exception of early neural crest migration) the underlying cell biological events are largely unexplored (Kulesa et al., 2004; Kulesa and Fraser, 1998).

Our current understanding of cell movements in craniofacial development stems largely from vital dye tracing experiments (Cerny et al., 2004; Hu et al., 2003; Lee et al., 2004; McGonnell et al., 1998; Serbedzija et al., 1992). As a result, studies usually characterize wholesale tissue movements rather than behavior of individual cells. Because live imaging of morphogenetic movements is extremely challenging, very little is known about coordinated cell behaviors and polarization during craniofacial development. In addition, we currently lack appropriate fluorescently-tagged mouse lines and are further hampered by the difficulties in performing long-term cultures of craniofacial tissues. A number of new studies, combined with advances in imaging techniques, are now beginning to examine the cell biology of craniofacial development, particularly in zebrafish. Understanding cell-cell interactions, how neighbor relationships change over time, and the cellular basis for morphogenetic processes remain a huge but essential challenge in the field.

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