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Micromanaging Palate Development

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Abstract

Development of the facial skeleton is one of the most intriguing and intricate events that occur during human development. Most of the bone, cartilage and connective tissue that compose the face and neck arise from a class of cells, referred to as neural crest cells, which are initially located at some distance from the facial primordium. A complex set of events regulated by specific gene products direct the formation, migration and differentiation of these cells, leading to what is viewed as “prototypical” adult facial features. These basic developmental processes are recapitulated during the formation of the palate, termed palatogenesis. In this review, we summarize the basic embryology leading to palate formation, discuss mechanisms that can lead to palatal dysmorphologies and highlight a new interaction that has recently been demonstrated to play a role in palate development. This interaction, involving small non-coding RNAs referred to as microRNAs, not only establishes a new level of regulation to cellular development, but may also serve as attractive targets for future efforts directed at clinical treatment of birth defect syndromes.

Embryology of palatal development: neural crest cell formation, migration and differentiation

During early development in all vertebrates, the neural tube (which will later form the brain and spinal cord) forms from the invagination of a sheet of neural ectoderm cells. The point at which the neural folds fuse is referred to as the neural crest since the tissue is elevated above the neural ectoderm; it is from here that neural crest cells (NCCs) arise (Fraser, Keynes and Lumsden, 1990; Le Douarin, Ziller and Couly, 1993; Lumsden, Sprawson and Graham, 1991). Around the time of neural tube closure, NCCs migrate away from the neural tube along specific pathways and give rise to a wide variety of tissues in the embryo along its entire rostral (head)-caudal (tail) axis. These cells are multipotent, in that one progenitor cell can give rise to a diverse array of cell types, including pigment cells (melanocytes) and intestinal neurons (Le Douarin, et al., 1993). However, only those NCCs in the head (referred to as cranial NCCs) have the endogenous potential to form bone and cartilage, in addition to other derivatives, including cranial neurons and pigment cells (Couly, Coltey and Le Douarin, 1993; Couly, Grapin-Botton, Coltey and Le Douarin, 1996; Kontges and Lumsden, 1996; Lumsden, et al., 1991; Noden, 1983, Noden, 1988). Indeed, most structures that comprise the facial skeleton (including the bone, cartilage, connective tissue and portions of the teeth) arise from NCCs (Chai and Maxson, 2006). Moreover, it has been suggested that the evolution of the vertebrate face has been driven in part by NCCs themselves.

In the head, NCCs migrate from the neural tube in three streams (emanating from the posterior forebrain, midbrain and hindbrain). These migratory cells move through the embryo and fill the pharyngeal arches, transient structures on the ventral embryo surface (Couly, et al., 1993; Fraser, et al., 1990; Le Douarin, et al. 1993; Lumsden, et al., 1991). Although it is thought that

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NCCs contain some positional information from where they came, it is the signals from the surrounding environment in which they end up that subsequently provide most of the patterning information to NCCs (Trainor, Melton and Manzanara, 2003). This information primes them toward differentiation into specific derivatives, with specific signals within each arch region dictating the cells' final developmental fate. These fates include the upper jaw, palate, lower jaw and cartilage of the throat. This developmental paradigm is highly conserved in most vertebrate species, including human, mouse and zebrafish, the three species that will be the focus of the remainder of this review

Palatogenesis: molecular mechanisms of normal and abnormal palate development

Palatogenesis gives rise to two distinct structures: the primary and secondary palates. The primary palate (part of the hard palate) is formed primarily through the fusion of the medial nasal prominences along the midline early in development (occurring between 7 and 10 weeks of gestation in humans; 11–12 days of gestation in the mouse). The secondary palate is composed of two palatal shelves that fuse from either side of the oral cavity (oropharynx) and form the remainder of the hard palate and all of the soft palate. The developmental origin of the secondary palate is arguably more complex than the primary palate and has been extensively studied in normal and mutant strains of mouse embryos. The mammalian palate is composed of both NCC-derived cells and the surrounding ectoderm (future skin or epithelium). Beginning at about embryonic day (E) 8.25 in the mouse (full gestation is 20 days), NCCs migrate to fill the space adjacent to the oral ectoderm, where they then receive patterning cues from the ectoderm. Beginning at E11.5, the NCC-derived cells form two downward projecting structures called palatal shelves. Over the next day, the shelves undergo tremendous growth, projecting downward on either side of the tongue, separating the tongue and oral epithelium. By E13.5, the shelves begin to ascend, due in part to the downward movement of the developing tongue, removing a physical barrier to palate elevation. A subset of NCC-derived cells within the palatal shelves begin to ossify at this stage, leading eventually to formation of the palatine bones (the bony (hard) part of the palate). At E14.5, the palatal shelves approach one another and fuse, resulting in a complete separation between the oral and nasal cavities. The fusion is accompanied by degeneration of the medial edge epithelial (MEE) cells, most likely through cell death. An identical process occurs in humans between 7 and 12 weeks of gestation. In zebrafish, the formation of the primary palate is similar to mammals, with NCCs migrating to the oral ectoderm along a structure called the optic stalk (bundles of axons exiting the eye). Because fish do not have a nasal cavity (or nasopharynx), secondary palatal formation does not occur. However, the molecular mechanisms that guide NCC migration and differentiation are highly conserved between most vertebrates, making both mouse and zebrafish valuable species in which to study palatogenesis (Kimmel, Walker and Miller, 2007).

Numerous events can disrupt palatogenesis, though the genetic basis for many of these in humans remains unknown. Over 80% of cleft palate cases in humans (without cleft lip) are considered “nonsyndromic” (occurring as an isolated abnormality) (Christensen and Mitchell, 1996), though this number may be considerably lower (Spritz, 2001). It has been difficult to define the genetic basis of cleft palate in humans, due to interaction of multiple genes and general genetic heterogeneity of humans. In contrast, while palatogenesis occurs via almost identical mechanisms in mice (and similar to what occurs in zebrafish), the power of genetics and the ability to produce genetically modified animals have led to the identification of numerous genes involved in different aspects of palate formation and have provided evidence later used to identify causative genes in human cleft palate cases (Schutte and Murray, 1999).

By examining different mouse strains in which genes have been inactivated, investigators have gained a more detailed understanding of the genetic pathways required for normal palate

development. As described above, the first event required for palatogenesis is proliferation and migration of NCCs to the area adjacent to the oral ectoderm. A failure of NCCs to reach the oral ectoderm results in an absence of palatal shelves (Fig. 1B). This is the basis for the palatal defects observed in a mouse model of Treacher Collins syndrome, where haploinsufficiency (loss of one gene copy) of the *Tcof1* gene in mice leads to a reduced number of migrating NCCs and hence cleft palate (Dixon, et al., 2006). In addition, in zebrafish containing a loss or reduction of the *sonic hedgehog (shh)* gene, NCC fail to migrate toward the future palatal area, resulting in loss and/or failure of fusion of the anterior palatal neurocranium (Wada, et al., 2005). The second crucial event required for palatogenesis is the elevation of the palatal shelves (Fig. 1C, D). Failure of shelf elevation can result from adhesion of the palatal shelves to the mandible or tongue (Fig. 1C). This appears to be the defect in mice containing a targeted mutation in the *fibroblast growth factor 10 (Fgf10)* gene (Alappat, et al., 2005). In addition, hypoplasia of tongue, which results in the tongue projecting upward rather than outward, can result in a physical obstruction to palatal shelf elevation (Fig. 1D). This type of change is observed in mice with a reduced level of the basic helix-loop-helix transcription factor Hand2 (Barbosa, et al., 2007; Yanagisawa, Clouthier, Richardson, Charité and Olson, 2003). In addition, loss of *Zfhx1a* results in a delay in shelf elevation, suggesting that elevation is a programmed event in which signals from the NCC-derived cells drive the process (Jin, Li, Higashi, Darling and Ding, 2008). The third event required for palatogenesis is the extension of the elevated shelves towards each other (Fig. 1E). Failure of palatal shelf growth is observed when expression of *Msx1* and *transforming growth factor beta receptor 2 (Tgfr2)* is reduced within the NCC-derived shelf (Ito, et al., 2003; Satokata and Maas, 1994). The fourth event required for palate development is the adhesion and fusion of the palatal shelves (Fig. 1F). In *Tgfr3* mutant mouse embryos, there appears to be an adhesion defect that prevents the two shelves from fusing (Taya, O'Kane and Ferguson, 1999; Tudela, et al., 2002), while in *Tgfr2*-deficient mice, the MEE cells fail to undergo apoptosis, blocking shelf fusion (Xu, et al., 2006). It is important to note that the genes described above have a variety of roles in other developmental events during embryogenesis in addition to the ones described for palatogenesis. Moreover, there are likely many genes involved in palatogenesis that are still unknown or who have unknown functions. From this description, it is clear that the regulation of palatogenesis is complex and still not well understood.

MicroRNAs: a special class of non-coding RNAs that regulate development processes

As described above, regulation of developmental processes occurs through intricate and continuous refinement of gene expression. One newly discovered mechanism for controlling gene expression is through the action of small non-coding ribonucleic acids (RNAs) referred to as microRNAs (miRNAs). These small non-coding regulatory RNAs are ~22 nucleotides in length (Lagos-Quintana, Rauhut, Lendeckel and Tuschli, 2001; Lee, Feinbaum and Ambros, 1993) and are thought to inhibit translation of specific target mRNAs by binding to a recognition sequence in the 3' untranslated region (UTR) of the target mRNA (Lee, et al., 1993; Wightman, Ha and Ruvkun, 1993). The first miRNA was discovered in the worm *Caenorhabditis elegans*, (*C. elegans*) where a gene, *lin-4*, known to be involved in the timing of *C. elegans* larval development, was found to encode a pair of small RNAs, though did not produce a subsequent protein (Lee, et al., 1993). Of the pair of RNAs (one 22 nucleotides long and the other 61 nucleotide long), the longer was found to fold into a stem loop and was also the precursor of the shorter one. Subsequently, it was discovered that *lin-4* expression resulted in a decrease in LIN-14 protein without a change in the quantity of *lin-14* mRNA (Wightman, et al., 1993). LIN-14 was also crucial for timing of *C. elegans* larval development, with the affect dependent on the dosage of *lin-14* (Ambros and Horvitz, 1987). It was thus determined that *lin-4* action was the mechanism controlling the level of *lin-14* mRNA and thus controlling

cell fate decisions. Since the discovery of *lin-4*, hundreds to thousands of putative miRNAs have been identified in multiple species (Bartel, 2004; Lagos-Quintana, et al., 2001; Song and Tuan, 2006).

miRNAs have been implicated in a wide variety of biological processes, including cell proliferation, cell death, the regulation of fat metabolism, insulin secretion, hematopoiesis, and stem cell maintenance and differentiation (Bartel, 2004; Song and Tuan, 2006). Additionally, genes for miRNAs are deleted in many cases of chronic lymphocytic leukemia (Calin, et al., 2002), with reduced levels of miRNAs also observed in colorectal cancer (Michael, O'Connor, van Holst Pellekaan, Young and James, 2003). However, much of the interest has centered on the role that miRNAs may play in developmental abnormalities. One example is the human gene *DGCR8*, which is involved in the processing of miRNAs (Gregory, et al., 2004; Han, et al., 2004). *DGCR8* is located on human chromosome 22q11, the region that is deleted in DiGeorge Syndrome (DGS), a congenital condition that results in defects in the heart, face and thymus, in addition to neurocognitive defects (Han, et al., 2004; Lindsay, 2001; Shiohama, Sasaki, Noda, Minoshima and Shimizu, 2003). This finding suggests that loss of normal miRNA function may in part lead to the DGS phenotype. It is also likely that many developmental events are regulated by multiple miRNAs expressed at different times during the developmental process. miRNA expression array analysis (a method to examine the expression of hundreds of miRNAs simultaneously) of mouse brain tissue has demonstrated that the miRNAs expression profile changes during brain development. In addition, dysregulation of certain miRNAs leading to severe developmental defects of the brain (Krichevsky, King, Donahue, Khrapko and Koslik, 2003). It therefore appears that the temporal and spatial specificity of miRNA expression is crucial for its function.

Regulation of gene expression by miRNAs takes place by two different mechanisms. For near perfect complementation between the miRNA and miRNA recognition sequence within a target mRNA 3' UTR, the target mRNA is cleaved by a complex associated with the miRNA and then degraded by the cell (Hutvagner, et al., 2001; Zheng and Cullen, 2003). After this cleavage occurs, the intact miRNA can recognize and facilitate the cleavage of additional messages. For targets in which the 3' UTR recognition sequence shows less complementation, translational repression is used to control gene expression (Olson and Ambros, 1999). There has also been a third mechanism proposed to control gene expression using deadenylation of the 3'-poly(A) tail and subsequent decay of the mRNA (Wu, Fan and Belasco, 2006).

Pdgfra signaling is required for palate development

While microRNAs are involved in numerous developmental events, until recently microRNA regulation of facial morphogenesis had not been described. However, Postlethwait and colleagues have recently taken advantage of zebrafish to show that miR-140 (mirn140) is required for normal migration of a subset of NCCs towards the oral ectoderm, where they later take part in palate development (Eberhart, et al., 2008). This finding came about from a study investigating the function of platelet-derived growth factor receptor alpha (*Pdgfra*) signaling in zebrafish facial development. The zebrafish offers a powerful model to study craniofacial development (Kimmel, Miller and Keynes, 2001; Schilling and Kimmel, 1997). Because of their rapid development and transparency, NCCs can be followed as they migrate into the pharyngeal arches. In addition, both forward and reverse genetic analysis has revealed many genes that have roles in the multistep process of craniofacial development.

Mouse embryos lacking the *Pdgfra* gene die near birth with rib and vertebrae defects, neural tube closure defects and midfacial and palatal clefting (Pickett, Olsen and Tallquist, 2008; Soriano, 1997; Tallquist, Weismann, Hellstrom and Soriano, 2000). Conditional inactivation of *Pdgfra* only in NCCs also leads to midfacial and palatal clefting, indicating that the function

of *Pdgfra* is cell autonomous to NCCs (Tallquist and Soriano, 2003). Postlethwait and colleagues found that in zebrafish, *pdgfra* was expressed in migrating NCCs. In contrast, *platelet-derived growth factor a* (*pdgfaa*, the gene encoding the ligand that binds to the *Pdgfra*) is expressed along the route that NCCs follow between the hindbrain and the facial ectoderm (we will refer to these specific NCCs as palatal precursor cells). High *Pdgfaa* levels were especially observed around the optic stalk, by which the palatal precursor cells migrate on their way to the oral ectoderm (Fig. 2A). In the *b1059* zebrafish strain that carries a mutation resulting in decreased *pdgfra* expression (referred to as a hypomorphic mutation; in contrast, a normal mutation would result in no *pdgfra* expression), palatal precursor cells fail to disperse after leaving the hindbrain, resulting in severe defects in the palatal neurocranium region. A similar phenotype was observed in zebrafish in which the *pdgfra* gene was knocked down using an antisense morpholino (a short oligonucleotide that blocks gene function) against *pdgfra*, suggesting that proper migration of palatal precursor cells to the oral ectoderm required *Pdgfra* signaling. These results implicate *Pdgfra* signaling as a potential chemoattractant. Complicating this scenario was the finding that overexpressing of *pdgfaa* in zebrafish resulted in bunching of NCCs around the optic stalk, already a prominent source of *Pdgfaa* for palatal precursor cells migrating to the oral ectoderm. It therefore appears that palatal precursor cells require *Pdgfra* signaling to reach the optic stalk, though this response must then be attenuated in order for the palatal precursor cells to reach the oral ectoderm.

MicroRNA 140 disconnects palatal precursor cell migration from *Pdgfra* signaling

The ability of palatal precursor cells to adjust their response to *Pdgfaa* rests with miR-140, a miRNA that is expressed along the entire migratory path of palatal precursor cells destined for the area adjacent to the oral ectoderm (Fig. 2A). miR-140 binds to the 3' untranslated region of the *pdgfra* mRNA and interferes with its translation into a functional protein; when overexpressed in zebrafish embryos, miR-140 produced a similar palatal defect to those observed in the *b1059* mutant, suggesting that the *pdgfra* mRNA is the target of miR-140. Further, morpholino knockdown of miR-140 resulted in bunching of palatal precursor cells near the optic stalk, suggesting that miR-140 is at least partially responsible for attenuating *Pdgfra* signaling in palatal precursor cells, thus allowing them to reach the oral ectoderm (Fig. 2B). However, the mechanism of miR-140 action is not completely clear. While *miR-140* is expressed along the entire migratory pathway, it appears to only act between the optic stalk and oral ectoderm. One explanation for this could be that NCCs have a temporal sensitivity to miR-140 action, with only late migrating palatal precursor cells being sensitive to miR-140 regulation. Another, more likely explanation is that only a subset of NCCs (specifically palatal precursor cells) rely on *Pdgfra* signaling as an attractant cue, thus limiting the utility of miR-140 action. This seems to be at least partially true, since only palatal precursor cells are affected by miR-140 overexpression or loss. Further analysis is required to determine the temporal requirement of miR-140 in NCC development.

Model Systems, microRNAs and palate development

As described above, development of the mammalian palate is a compilation of different developmental processes within the craniofacial region. The outcome of these events is a separation of the oral and nasal cavities. Since fish do not have a nasal cavity, they do not have a true secondary palate. So how can different vertebrate models of cleft palate be used to understand the molecular regulation of palatogenesis? The answer lies in the conserved nature of the signals that regulate facial development, including the palatal precursor cells (Clouthier and Schilling, 2004; Yelick and Schilling, 2002). Furthermore, the location of miR-140 is highly conserved as is the miR-140 binding site in the 3' UTR of the *Pdgfra* gene. This argues that, like other conserved regulatory events in facial morphogenesis, microRNA regulation of

palatal development may be a conserved developmental mechanism among a wide variety of vertebrates.

Future Directions of miRNA regulation of palatal development

As the function of many miRNAs remains elusive, it should not be surprising if other miRNAs besides miR-140 are found to be involved in guiding the development of palatal precursor cells. miRNA microarrays are now being used to create miRNA “expression profiles” in specific tissues that can then be compared with the expression profile of miRNAs in different disease or developmental states. Such an approach has recently been used to identify miRNAs that act as metastasis suppressors in human breast cancers (Tavazoie, et al., 2008). Analyzing miRNA expression during normal and abnormal palatogenesis in mouse and zebrafish cleft palate models should prove equally informative and provide an exciting opportunity to elucidate the mechanism by which identified miRNAs act. While current details about the mechanisms leading to palatal defects are still valid, miRNAs will extend our understanding of the molecular control of palate development. In addition, miRNAs may one day provide a therapeutic approach to treat cleft palate. One can envision blocking inappropriate miRNA function or overexpressing missing miRNAs to recapitulate normal developmental events. Such approaches will not be easy, as most molecular events in human palatogenesis occur during early pregnancy. However, it is not unrealistic to envision that miRNAs will be useful in correcting palatal defects later in pregnancy when they can be used in combination with a tissue engineering approach (such as one using an implantable scaffold matrix to bridge a secondary cleft palate). Indeed, while a diagnosis of cleft palate can be devastating to expectant parents, miRNAs hold a treatment possibility that only a few short years ago was unimaginable.

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A. Palatal shelves before elevation **B. Failure of palatal shelf formation** **C. Failure of shelf elevation: fusion** **D. Failure of shelf elevation: tongue** **E. Failure of palatal shelf growth** **F. Failure of palatal shelf fusion** **Figure 1. Types of defects that can lead to cleft secondary palate**

The normal early palate is shown in A (approximately E12.5 in the mouse). In B–F, defects affecting different stages of palatogenesis between E12.5–E14.5 are shown. The type of defect is listed above each figure. Modified from Chai and Maxson (2006).

Figure 2. Blocking miR-140 action prevents palatal precursor cells from reaching the oral ectoderm
A. In wild type zebrafish embryos, *Pdgfra* and *miR-140* are expressed by NCCs, while *Pdgfra* is found in the environment through which the NCCs migrate. **B.** Blocking *miR-140* expression in zebrafish through the use of a morpholino leads to clumping of NCCs (palatal precursor cells) around the optic stalk, with very few able to reach the area adjacent to the oral ectoderm. Modified from Clouthier (2008).