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Palate Morphogenesis: Current Understanding and Future Directions

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Abstract

In the past, most scientists conducted their inquiries of nature via inductivism, the patient accumulation of “pieces of information” in the pious hope that the sum of the parts would clarify the whole. Increasingly, modern biology employs the tools of bioinformatics and systems biology in attempts to reveal the “big picture.” Most successful laboratories engaged in the pursuit of the secrets of embryonic development, particularly those whose research focus is craniofacial development, pursue a middle road where research efforts embrace, rather than abandon, what some have called the “pedestrian” qualities of inductivism, while increasingly employing modern data mining technologies. The secondary palate has provided an excellent paradigm that has enabled examination of a wide variety of developmental processes. Examination of cellular signal transduction, as it directs embryogenesis, has proven exceptionally revealing with regard to clarification of the “facts” of palatal ontogeny—at least the facts as we currently understand them. Herein, we review the most basic fundamentals of orofacial embryology and discuss how functioning of TGF β , BMP, Shh, and Wnt signal transduction pathways contributes to palatal morphogenesis. Our current understanding of palatal medial edge epithelial differentiation is also examined. We conclude with a discussion of how the rapidly expanding field of epigenetics, particularly regulation of gene expression by miRNAs and DNA methylation, is critical to control of cell and tissue differentiation, and how examination of these epigenetic processes has already begun to provide a better understanding of, and greater appreciation for, the complexities of palatal morphogenesis.

Keywords

palate; morphogenesis; orofacial; growth factors; signal transduction; epigenetics

INTRODUCTION

Nothing in Nature is more fascinating than the development of a complex, multicellular organism that progressively acquires the essential attributes of its parents. When one considers the complexity of molecular, cellular, and tissue interactions that are necessary to orchestrate craniofacial morphogenesis, one must indeed be amazed at Nature’s handiwork. However, despite the fact that most infants are born normal, orofacial clefts are seen with an alarming frequency of 1–2 in 1000 live births and represent nearly one-half of all craniofacial anomalies (Stanier and Moore, 2004). Indeed, 1% of infants born worldwide (1 million) each year exhibit some form of facial dysmorphology. Most dramatic is the

observation that in this country alone, a baby is born with a facial cleft every hour, of every day of the year! Orofacial clefts are customarily described as those encompassing the lip and/or palate (CL/P), and those affecting only the palate (CPO). While orofacial clefts frequently are seen as part of a syndrome, the majority are isolated, without other accompanying anomalies (Gorlin et al., 2001).

While morphogenesis of the facial complex has been well described, new insights continue to refine our appreciation of orofacial ontogeny. As such, the cast of genes and proteins that contribute to the choreography of orofacial development continues to expand. The National Center for Biotechnology Information's (NCBI) Online Mendelian Inheritance in Man (OMIM) currently lists nearly 6000 gene loci that have been identified as being relevant to inherited human diseases—20% of these are candidates associated with craniofacial/oral diseases and disorders. With over 200 described craniofacial syndromes, remarkable progress has been made in recent years with regard to identifying genetic culprits responsible for various craniofacial disorders. Genes responsible for these disorders have either been mapped to a chromosome or actually isolated and identified in over 50 syndromes. Nevertheless, for the vast majority of these birth defects, including most conditions of oro/facial clefting, the underlying molecular events responsible persist in eluding our complete understanding. What we do know, however, is that normal orofacial morphogenesis requires extensive reciprocal signaling between numerous embryonic tissues. The secondary palate, in particular, has provided an excellent paradigm that has enabled examination of cellular signaling-mediated processes regulating embryonic development. These include, but are not limited to, cell proliferation, extracellular matrix (ECM) metabolism, epithelial-mesenchymal transformation, apoptosis, cell migration, and cellular signal transduction mechanisms.

In 1662, when the Royal Society was founded, there were only two scientific journals. The number reached 100 by 1850 and 1000 by 1900. In 1950, there were over 10,000 scientific journals. At last count, the number exceeded 100,000! A search of PubMed using the search phrase “palate development” resulted in nearly 4500 publications since the 1950s. Thus, we can by no means be inclusive. Nor is it our intent in this review to be selectively biased. Rather, we have attempted to grasp the difference between the essential and the peripheral by carefully selecting from the extant literature the essential elements of our current knowledge regarding the functionality of specific genes and gene families in orofacial development, focusing on critical signal transduction pathways. The rationale for this approach is based on the fact that our understanding of the mosaic of interacting signal transduction pathways associated with normal craniofacial development has expanded significantly in the past several years. Excellent reviews exist that focus on areas not covered by our survey (Schutte and Murray, 1999; Lidral and Murray, 2004; Tapadia et al., 2005; Dhulipala et al., 2006; Gritli-Linde, 2007; Lidral et al., 2008; Jugessur et al., 2009). By concentrating on the functionality of specific genes and signaling pathways, we hope to provide enlargement of our understanding of orofacial ontogeny and provide clarification of how the embryo makes sense of the apparent ceaseless chatter of intra- and intercellular developmental signals to which it is exposed.

OROFACIAL EMBRYOLOGY

The facial region in mammalian embryos develops primarily from the frontonasal prominence (forehead, middle of the nose, philtrum of upper lip, and primary palate), the lateral nasal prominences, and the maxillomandibular prominences of the first branchial arch (maxilla, mandible, lateral portions of upper lip, and secondary palate) (see Fig. 1). Merging of superficially separate facial prominences gives rise to the upper lip (see Fig. 1), whereas fusion of completely separate tissue processes gives rise to the secondary palate (see Fig. 2).

As with all of the branchial arches, the first branchial arch contains mesoderm-derived mesenchymal cells, a cartilaginous core, its own cranial nerve (the trigeminal), a blood vessel (an aortic arch), and neuroectoderm-derived neural crest cells. The reader is referred to three excellent overviews of branchial arch development (Graham, 2003; Helms and Schneider, 2003; Grevellec and Tucker, 2010). In brief, orofacial development is dependent, in part, on the migration of neural crest cells derived from the neuroectoderm of rhombomeres 1–3 (Kontges and Lumsden, 1996) into the first two branchial arches (see Fig. 3), and diversification of neural crest cell fates (LaBonne and Bronner-Fraser, 1999; Jheon and Schneider, 2009). Cranial neural crest cells have been shown capable of directing their own morphogenesis (Schneider and Helms, 2003; Tucker and Lumsden, 2004), as well as being responsive to autocrine/paracrine signals that are thought to provide critical developmental cues as these cells contribute to orofacial ontogenesis (Golding et al., 2000; Couly et al., 2002; Graham et al., 2005; Helms et al., 2005; Noden and Trainor, 2005). Functional importance of the neural crest is put into sharp focus by the observation that many craniofacial malformations are thought to be due to abnormal neural crest cell generation, migration, proliferation, and/or survival (Walker and Trainor, 2006; Passos-Bueno et al., 2009).

Several excellent reviews of craniofacial morphogenesis have appeared in recent years (Francis-West et al., 2003; Helms et al., 2005; Tapadia et al., 2005; Chai and Maxton, 2007). In this overview, we have chosen to focus on certain, but by no means all, factors generally accepted as playing a pivotal role in development of the orofacial region, and specifically, the secondary palate. The palatal processes originate as bilateral extensions from the oral aspect of the maxillary processes, make contact and fuse with one another along their anterior–posterior length giving rise to the secondary palate or roof of the oral cavity (see Fig. 2). Significantly, abnormal development of this region often results in orofacial clefts which can manifest themselves in isolation, or as part of a syndrome, such as in Apert, Roberts, Stickler, and Treacher Collins, among others.

PALATE MORPHOGENESIS: TRANSFORMING GROWTH FACTORS β

For an excellent overview of the contributions of the transforming growth factor β (TGF β) family of cytokines in craniofacial development, the reader is referred to Dudas and Kaartinen (2005). In brief, the TGF β s exhibit discrete spatio-temporal patterns of expression in the developing orofacial region (Fitzpatrick et al., 1990; Gehris et al., 1991; Pelton et al., 1991; Behman et al., 2005) (see Fig. 4), and their expression levels can be modulated by numerous convergent signaling peptides (Gehris et al., 1994; Potchinsky et al., 1996; Nugent et al., 1998). Embryonic orofacial tissue contains functional TGF β receptors (Cui and Shuler, 2002; Linask et al., 1991; Dudas et al., 2006; Nakajima et al., 2007) that, when activated, elicit changes in orofacial cell proliferation (Linask et al., 1991) as well as synthesis (D'Angelo and Greene, 1991; D'Angelo et al., 1994) and remodeling (D'Angelo et al., 1994; Brown et al., 2002) of the ECM. The importance of the ECM to palatal development has been recognized since the founding of the Teratology Society in 1961 (Walker, 1961). Long thought to provide a “motive force” for palatal shelf movement (Morris-Wiman and Brinkley, 1992), it continues to remain unclear precisely how extracellular components within the palatal mesenchyme orchestrate movement of the palatal processes (Carinci et al., 2007). What is known is that matrix metalloproteinase (MMP)-mediated remodeling of the ECM plays an essential role in both morphogenetic movement of the palate (Mansell et al., 2000; Morris-Wiman et al., 2000; de Oliveira Demarchi et al., 2010), as well as palate epithelial differentiation (Miettinen et al., 1999) and fusion (Blavier et al., 2001). Indeed, an association between a polymorphism in the *MMP3* and palatal clefting has been observed (Letra et al., 2007). Evidence for the functional role played by the TGF β family of cytokines in orofacial development comes from the cleft

palate exhibited by murine embryos, in which *TGFβ2* or *TGFβ3* were deleted by homologous recombination (Karttinen et al., 1995; Proetzel et al., 1995; Koo et al., 2001) and the demonstrated role of *TGFβ* in orofacial tissue differentiation (D'Angelo and Greene, 1991; Gehris and Greene, 1992; Brunet et al., 1993; D'Angelo et al., 1994; Cui et al., 2003; Ahmed et al., 2007; Martinez-Sanz et al., 2008).

The highly conserved Smad proteins transduce *TGFβ* signaling from the plasma membrane to the nucleus. Numerous excellent recent reviews outline molecular mechanisms underlying various aspects of this signal transduction process (Hill, 2009; Moustakas and Heldin, 2001; Wrighton et al., 2009; Zhang, 2009). Elements of the Smad component of the *TGFβ* intracellular signaling system have been identified and characterized in cells of the embryonic orofacial region (Greene et al., 2003; Xu et al., 2008). Targeted disruption of *Smad* genes in mice substantiates their importance in craniofacial development. *Smad2* heterozygous null mutants exhibited cleft palate, mandibular hypoplasia and cyclopia (Nomura and Li, 1998), and neural crest specific inactivation of the *Smad4* gene demonstrated its requirement for cranial neural crest cell survival in the first branchial arch (Ko et al., 2007). Moreover, the cleft palate phenotype in *TGFβ3*^{-/-} mice was rescued by overexpression of a *Smad2* transgene in palatal medial edge epithelial (MEE) cells (Cui et al., 2005). In addition, haploinsufficiency of the Smad binding protein 1 gene (*Smadip1*) has been identified as contributing to a Hirschsprung-like syndrome exhibiting facial dysmorphology (Cacheux et al., 2001). The primary role of Smads, however, is not to target specific genes solely via DNA binding, but rather to function as comodulators of transcriptional activity by differentially associating with a wide spectrum of nuclear proteins, thus eliciting both positive and negative regulation of numerous genes (Attisano and Wrana, 2000; ten Dijke et al., 2000).

Multiple members of the *TGFβ* superfamily of signaling molecules exhibit differential and temporal expression patterns during orofacial development. Using high-density microarrays to examine mRNA profiles in developing murine secondary palatal tissue, the expression of 26 members of the *TGFβ* superfamily was found to be significantly altered by ≥1.5-fold (Table 1) (Mukhopadhyay et al., 2006a, b). Members of this family elicit a wide variety of downstream biological actions. In developing craniofacial tissue, these include, but are not limited to, tissue morphogenesis (Oka et al., 2007; Zouvelou et al., 2009), cell proliferation (Linask et al., 1991; Iwata et al., 2010), cell differentiation (Matsunobu et al., 2009), apoptosis (Rawlins and Opperman, 2008), and ECM synthesis (D'Angelo et al., 1994; Brown et al., 2002). While the full range of roles in orofacial development for these cytokines has not yet been completely revealed, it is clear that crosstalk with other signal transduction pathways (Weston et al., 1998; Baroni et al., 2006), plays a central role in morphogenesis, growth, and cell differentiation during normal orofacial development.

PALATE MORPHOGENESIS: BONE MORPHOGENETIC PROTEINS

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta superfamily, whose expression, like that of the *TGFβs*, is pronounced during development of embryonic/fetal orofacial tissue (Francis-West et al., 2003; Bennett et al., 1995; Gong and Guo, 2003; Xu et al., 2008) wherein they orchestrate an extensive array of biological responses. For example, BMP signaling has been implicated in migration of the cranial neural crest cells into the first branchial arch (Dudas et al., 2004; Nie et al., 2006), outgrowth of facial primordia (Ashique et al., 2002; Shuman and Gong, 2007), fusion of the maxillary and nasal processes during formation of the upper lip (Liu et al., 2005), formation of the secondary palate (Zhang et al., 2002; Levi et al., 2006; Thomason et al., 2008), epithelial-mesenchymal interactions leading to orofacial bone and cartilage formation (Bennett et al., 1995) and tooth development (Tompkins, 2006). Moreover, targeted

disruption of genes encoding BMPs, BMP receptors, and their downstream signal transducers has revealed that BMP signaling is requisite for normal orofacial development (Zhao, 2003).

Several of the aforementioned BMP-regulated developmental processes are thought to be mediated by activation of the homeobox transcription factors *Msx1* and *Msx2*, particularly as they relate to craniofacial development (Alappat et al., 2003). Indeed, functionality of the BMPs in orofacial development is supported by the observation that expression of *Bmp2* and *Bmp4* in embryonic palatal mesenchyme requires the expression of the *Msx1* homeobox gene (Zhang et al., 2002), and that mutations in *Msx1* are associated with nonsyndromic cleft palate and tooth agenesis in humans (van den Boogaard et al., 2000). Furthermore, transgenic expression of *Bmp4* in *Msx1*^(-/-) murine embryonic palatal mesenchyme rescues the cleft palate phenotype (Zhang et al., 2002). Moreover, polymorphisms within the *Msx1* locus are thought to contribute to the incidence of nonsyndromic forms of CLP (Lidral et al., 1998; Modesto et al., 2006).

Using a BMP-specific transcriptional reporter (BRE-luc) that can be dose-dependently activated following BMP treatment (Korchynskyi and ten Dijke, 2002), elements of the Smad component of the BMP intracellular signaling system were identified and characterized in cells derived from the embryonic orofacial region, and functional activation of the Smad pathway in these cells was demonstrated (Mukhopadhyay et al., 2008) (see Fig. 5). The inhibitors of differentiation (Id) family of helix-loop-helix (HLH) factors are BMP target genes (Hollnagel et al., 1999) that function as dominant negative inhibitors of HLH protein-dependent transcription (Iavarone and Lasorella, 2006) and subserve important roles during development (Ruzinova and Benezra, 2003). Upregulated by BMP2 or BMP4 in murine embryonic maxillary mesenchymal cells (Mukhopadhyay et al., 2006a), Id1, Id2, and Id3 represent inducible transcription factors that may play an important role in the regulation of apoptosis, cell proliferation, and cell and tissue differentiation (Norton, 2000)—all processes essential for normal orofacial development. Indeed, Id1 has been shown to act as a downstream target of BMP signaling during development of the secondary palate (Rice et al., 2005).

A detailed transcriptional map of BMP2 and BMP4 responsiveness in embryonic maxillary mesenchymal cells has also been delineated and offers revealing insights into crucial molecular regulatory mechanisms employed by these two growth factors in orchestrating embryonic orofacial cellular responses (Mukhopadhyay et al., 2006). Like the TGFβs, BMPs exert their effects by first binding to cell surface serine/threonine kinase receptors (Nohe et al., 2004), activation of which initiates Smad-mediated intracellular signaling cascades resulting in translocation of Smads 1, 5, and 8 into the nucleus (Sieber et al., 2009). Transcriptional complexes containing these Smads then bind to Smad-binding elements in the promoters of BMP target genes, thus regulating their expression (Miyazono et al., 2005). The functional importance of BMP signaling is emphasized by the fact that perturbation of *Bmp* expression (Lu et al., 2000), or inactivation of genes encoding various cell surface BMP receptors or their downstream Smad mediators (Chang et al., 1999; Dudas et al., 2004; Liu et al., 2005), results in a spectrum of orofacial malformations including cleft palate. Collectively, these data support the position that members of the BMP family play pivotal roles in development of the orofacial complex during embryogenesis.

Nature loves multifunctionality as a means to achieve efficiency. As such, BMP-induced receptor activation can also actuate the activity of mitogen-activated protein kinase (p38, JNK, and ERK) (Gallea et al., 2001; Guicheux et al., 2003), and PI3 kinase and PKC pathways (Kishigami and Mishina, 2005). What role these non-canonical BMP-mediated

signaling cascades play in craniofacial development is unknown but represents an area ripe for exploration.

PALATE MORPHOGENESIS: SONIC HEDGEHOG

Sonic hedgehog homolog (Shh) is the best studied ligand in the mammalian hedgehog signaling pathway. Acting as a morphogen, Shh regulates embryonic patterning of many systems during vertebrate organogenesis (Dorus et al., 2006). Proper spatio-temporal expression of sonic hedgehog (*Shh*) in embryonic facial ectoderm, neuroectoderm and pharyngeal endoderm during orofacial development has come to be recognized as critical for normal craniofacial morphogenesis (Cordero et al., 2004), especially outgrowth of the frontonasal process (Jeong et al., 2004; Marcucio et al., 2004) (see Fig. 6). Underexpression of *Shh* perturbs proper growth and development of the frontonasal and maxillary processes resulting in facial clefting, holoprosencephaly and cyclopia, while overexpression of *Shh* leads to a wider than normal gap between the eyes, a condition known as hypertelorism (Hu and Helms, 1999). Perturbations in certain Shh-related genes, important in midline patterning of the brain (Britto et al., 2002a, b; Hayhurst et al., 2008), are also associated with failure of the embryonic prosencephalon to divide into paired cerebral hemispheres resulting in holoprosencephaly (Marini et al., 2003; Cordero et al., 2004; Huang et al., 2007; Bertolacini et al., 2010). Since phenotypes vary, often including median cleft lip and other facial anomalies, it is thus not surprising that *Shh* also contributes to proper development of the facial skeleton (Jeong et al., 2004; Melnick et al., 2005).

Crosstalk during development between the Shh and BMP signaling pathways has been well documented (Liem et al., 2000; Zhang et al., 2000; Yuasa et al., 2002; Hornik et al., 2004; Bastida et al., 2009). *Shh*, normally expressed in embryonic palatal epithelial tissue (Rice et al., 2006), activates *Bmp2*, *Bmp4*, and *Fgf10* expression in the palatal mesenchyme, which in turn act as mitogens, presumably through maintenance of cyclin D1 and D2 expression, to stimulate mesenchymal cell division (Lan and Jiang, 2009). The importance of proper BMP-regulation of mesenchymal proliferation in palate development is highlighted by the observation that transgenic expression of *Bmp4* in *Mx1*-deficient mice rescued the cleft palate phenotype as well as restored *Shh* and *Bmp2* expression and normal levels of mesenchymal cell proliferation (Zhang et al., 2002). Further evidence for a developmental role for Shh in palatal epithelium comes from an animal model of nevoid basal cell carcinoma syndrome (NBCCS). NBCCS is a complex disorder characterized in part by facial dysmorphology, including cleft lip and palate. Causative mutations for NBCCS occur in the gene which encodes the principle receptor for the Hedgehog signaling pathway. A transgenic mouse model of NBCCS, expressing *Shh* in basal epithelium, exhibits, among other phenotypes, a cleft palate due to Shh-induced perturbation of palatal MEE differentiation (Cobourne et al., 2009). Thus, an exquisite reciprocal signaling between embryonic palatal epithelium and mesenchyme directs palatal shelf growth and morphogenesis as well as epithelial differentiation. *Shh*, expressed in the palatal epithelium, activates *Bmp2*, *Bmp4*, and *Fgf10* expression in the palatal mesenchyme (Lan and Jiang, 2009). FGF, in turn, signals back to the epithelium—where their receptors are specifically expressed (Lee et al., 2001)—to regulate epithelial expression of *Shh* (Rice et al., 2004; Welsh et al., 2007). Noteworthy is the fact that *Fgf10* homozygous null mutant mice exhibit cleft palate (Alappat et al., 2005), and mutations in these ligands or their receptors have been associated with syndromes characterized by craniofacial defects, including cleft palate, such as Apert, Saethre-Chotzen, and Crouzon, (Baroni et al., 2002; Britto et al., 2002a, b; Chun et al., 2002; Frank et al., 2002; Fujisawa et al., 2002; Shotelersuk et al., 2003; Snyder-Warwick et al., 2010). An overview of developmental syndromes involving orofacial clefting associated with FGF genes can be found in an excellent review by Pauws and Stanier (2007). Indeed, the FGF signaling pathway may contribute to as much as 3–5% of

nonsyndromic cleft lip/palate (Riley et al., 2007). *Shh* signaling therefore plays a central role in coordinating the reciprocal epithelial-mesenchymal interactions between FGFs, BMPs, various cyclins and specific Fox transcription factors (Lan and Jiang, 2009) that appear essential for normal palatal ontogeny.

PALATE MORPHOGENESIS: Wnt SIGNALING

Wnt proteins form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during development (van Amerongen and Nusse, 2009). The canonical Wnt signaling pathway is initiated by ligand binding to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled family proteins and ultimately resulting in a change in the amount of the multifunctional, nucleocytoplasmic transcription cofactor β -catenin that reaches the nucleus to mediate transcription (Mulholland et al., 2005). The cellular responses to Wnt signals include alterations in gene transcription (generally referred to as the canonical pathway), regulation of cell polarity, and changes in intracellular Ca^{2+} levels. Thus, Wnt stimulation can lead to at least three different signaling outputs. A current list of known Wnt genes and sequence alignments, as well as links to recent reviews on Wnt signaling can be found on the Wnt homepage (<http://www.stanford.edu/~rnusse/wntwindow.html>).

These secreted glycoproteins act as morphogens during early embryonic patterning, cell proliferation, differentiation, and apoptosis, and loss of Wnt function in vertebrates results in a wide spectrum of developmental defects (Wodarz and Nusse, 1998). Their role in craniofacial development has been documented (Cobourne and Sharpe, 2003; Yanfeng et al., 2003). The Wnt family of proteins has emerged as an essential contributor to neural crest cell induction, migration, and differentiation (Vallin et al., 2001; Schmidt and Patel, 2005). The currently prevailing notion is that neural crest cell induction involves interactions between a BMP gradient (established by the BMP antagonist Noggin), and FGF and Wnt signaling (Baker and Bronner-Fraser, 1997; LaBonne and Bronner-Fraser, 1999; Garcia-Castro et al., 2002; Raible and Ragland, 2005). While the canonical Wnt signaling pathway is required for neural crest induction, the noncanonical Wnt signaling pathway (planar cell polarity or Wnt- Ca^{2+}), via Wnt 11, appears to be required for neural crest migration (de Calisto et al., 2005). Migration of cranial neural crest cells into the first branchial arch, where they contribute to the development of orofacial skeletal components, also depends, in part, on Wnt signaling (Ikeya et al., 1997).

Cooperative interaction between the BMP and Wnt signaling pathways in developing orofacial tissue exists. Evidence for this comes, in part, from the observation that both BMP-2 and -4 upregulate the expression of genes encoding several frizzled proteins (Fzd-4, -7, and -9) (Mukhopadhyay et al., 2006b). Indeed, expression of *WNT-5a*, *-10a*, *-10b*, and *-11* genes has recently been detected in the mesenchyme of developing murine facial primordia where genes encoding Wnt-3, -4, -5a, and -10b, and Frizzled-related proteins (Fzd-4, Flamigo-1, secreted frizzled related sequence protein 4 or Sfrp4) were found to be differentially expressed (Mukhopadhyay et al., 2004). Moreover, expression analysis of Wnt genes during avian facial morphogenesis revealed that all regions of the developing face were capable of activating the canonical Wnt pathway (Geetha-Loganathan et al., 2009). Of the 19 known members of the Wnt family, 12 were found to be expressed in unique spatio-temporal patterns in murine embryonic palatal tissue during key phases of its development (Warner et al., 2009). For example, in situ hybridization with a *Wnt-2* specific riboprobe demonstrated no detectable expression in palatal tissue on gestational day 12.5, whereas on gestational days 13.5 and 14.5, expression of *Wnt-2* was detected in the palatal mesenchyme in a discrete spatial pattern (see Fig 7).

Evidence for the functionality of Wnt signaling in orofacial development comes from the observation that mutations in many Wnt genes have been associated with cleft lip with or without cleft palate (Niemann et al., 2004; Juriloff et al., 2006; Chiquet et al., 2008). Some of these Wnt family members exhibit expression patterns consistent with a role in regulation of midfacial development and lip fusion (Lan et al., 2006; Warner et al., 2009). Indeed, one of these Wnt family members-*Wnt5a*-whose deficiency results in cleft palate, appears to be requisite for palate mesenchymal cell proliferation and directional cell migration (He et al., 2008). Moreover, the demonstration of functional interactions between the Wnt and TGF β (Warner et al., 2005a, b) and Wnt and FGF (Lee et al., 2008) signaling pathways in cells of the embryonic orofacial region suggests that Wnt activation of the canonical pathway is an important mediator of first branchial arch mesenchymal cell growth and epithelial differentiation.

PALATE EPITHELIAL DIFFERENTIATION

As previously described, human and murine palatal processes, arise as bilateral extensions from the oral aspect of the maxillary processes, reorient from a vertical position lateral to the tongue, to a horizontal position above the tongue, making epithelial contact along the anterior-posterior length of the palate. Contact between homologous MEE results in the formation of what has been referred to as the MEE seam which is eventually removed to give rise to the single, continuous secondary palate, or roof of the oral cavity (see Fig. 2). How the MEE is eliminated has been the object of a significant amount of research interest. Two processes have emerged as the most likely means of MEE removal-apoptosis and transformation of the MEE into mesenchymal cells (EMT). Evidence exists for each of these processes and various laboratories have championed either apoptosis or EMT as the primary process leading to MEE removal.

During the 1960s and 1970s ultrastructural, autoradiographic and histochemical evidence offered compelling documentation of MEE cell death during palate development (Farbman, 1968; Hudson and Shapiro, 1973; Hassell, 1975; Pratt and Martin, 1975; Pratt and Greene, 1976; Greene and Pratt, 1978). More recently, the use of 3'-nick end labeling of dUTP (TUNEL) to detect apoptosis and immunohistochemistry and confocal microscopy to detect the presence of macrophages have confirmed that rodent MEE cells undergo cell death and that dead cells are phagocytosed by macrophages (Taniguchi et al., 1995; Martínez-Alvarez et al., 2000).

TGF β s are thought to be key regulators of MEE differentiation since murine embryos, in which *TGF β 2* or *TGF β 3* are deleted by homologous recombination (Kaartinen et al., 1995; Proetzel et al., 1995), exhibit cleft palate. Indeed, both MEE cell cycle arrest, and the induction of MEE apoptosis have been shown to be TGF β -dependent processes (Ahmed et al., 2007). Targeted disruption of Smad genes in mice substantiates their importance in craniofacial development inasmuch as *Smad2* heterozygous null mutants exhibit cleft palate, mandibular hypoplasia, and cyclopia (Nomura and Li, 1998). Moreover, *Smad2* siRNA transfection into palatal tissues in vitro prevented the removal of MEE (Shiomi et al., 2006). Furthermore, the cleft palate phenotype in *TGF β 3*^{-/-} mice can be rescued by overexpression of a *Smad2* transgene in palatal MEE cells (Cui et al., 2005). More recently, it has been shown that a TGF β target gene, *betaig-h3*, plays an important role in mediating MEE apoptosis (Choi et al., 2009a, b). Blocking *betaig-h3* expression with antisense oligodeoxynucleotides resulted in failure of MEE apoptosis and cleft palate in treated mice.

In recent years, the notion of disappearance of the MEE solely by cell death has been questioned and the process of transformation of the MEE into mesenchymal cells has gained much support. In an early study, electron micrographic analysis revealed loss of epithelial

characteristics, and gain of fibroblast-like features by MEE cells (Fitchett and Hay, 1989). In the years that followed, numerous studies presented evidence consistent with epithelial-mesenchymal transformation (EMT) of the MEE rather than programmed cell death. Using either carboxyfluorescein or vital Dil-labeling cell marking techniques to trace the fate of the MEE cells during palatal fusion, transition of labeled cells from an epithelial phenotype to a mesenchymal phenotype was demonstrated both in vitro (Shuler et al., 1991; Kang and Svoboda, 2002) and in vivo, (Shuler et al., 1992). Consistent with such transformation is the observation that the expression of both syndecan-1 and E-cadherin, two molecules shown to promote the epithelial phenotype, is abruptly lost when EMT of the palate MEE occurs in vivo (Sun et al., 1998a, b). More recently, utilization of the Cre/lox system to genetically mark *keratin-14*-expressing palatal epithelial cells has resulted in conflicting results. The Cre transgene, originally expressed only in the MEE, was reported to be both extensively expressed (Jin and Ding, 2006a), or entirely absent (Vaziri et al., 2005) in palatal mesenchyme during and after palate shelf fusion. Indeed, acceptance of EMT as a mechanism for the removal of palatal MEE has been highly varied in published studies (see Dudas et al., 2007 for extensive review).

Similar to its involvement in MEE apoptosis (see above), evidence suggests that TGF β signaling plays a role in EMT of the MEE. TGF β 3 is the main inducer of palatal shelf fusion and activates the key EMT transcription factors Lef1, Twist, and Snail1 in the MEE before actual EMT (Yu et al., 2008, 2009). In isolated and cultured MEE, phospho-Smads 2 and -4 were shown to activate lymphoid enhancer factor 1 (LEF1), which in turn inhibited expression of the E-cadherin gene thought to be responsible for breaking the MEE seam into small epithelial islands (Nawshad et al., 2007). LEF1 and Smad4 were also found to be necessary for upregulation of the mesenchymal markers vimentin and fibronectin.

While some investigators feel that removal of the MEE occurs exclusively via cell death (Cuervo and Covarrubias, 2004), many concede that a combination of apoptosis and EMT contribute to MEE removal. Indeed, evidence exists that MEE cells undergo apoptosis, even though they transform into mesenchymal cells (Taniguchi et al., 1995). A recent and extensive review of the literature supporting EMT of the MEE (Dudas et al., 2007) concluded that "...dye-based labeling and expression vector-based cell lineage tracing within the palatal epithelium have not yielded any conclusive, convincing evidence for epithelial to mesenchymal transition in the MEE." The controversy continues.

EPIGENETICS: miRNAs

Numerous genetic approaches involving both human populations and animal models have advanced the identification of genes involved in orofacial clefting (Spritz, 2001; Lidral and Murray, 2004; Jugessur and Murray, 2005; Scapoli et al., 2008; Jugessur et al., 2009). Much emphasis has been placed on the search for single-nucleotide polymorphisms (SNPs), where there is a DNA sequence variation, deletion or addition of a single nucleotide, within a gene. There are over 15 million SNPs in the human genome. A catalog of common genetic variants that occur in human beings, called the HapMap, describes these variants and the location in DNA where they occur. This information is being used to link genetic variants to the risk for specific diseases and should continue to prove invaluable for defining risk factors for craniofacial defects.

It has, however, become increasingly apparent that the genome is also organized in an entirely different plane, "above" the level of DNA sequence-at the epigenetic level. Epigenetics is the study of inherited changes in phenotype or gene expression via mechanisms other than changes in DNA sequence. Specific epigenetic regulated processes include interaction between two alleles of a single locus, transmission of cellular memory,

imprinting, X chromosome inactivation, gene silencing and, of most importance to the current discussion, tissue-specific cellular differentiation. Scientists globally have begun contributing to the Human Epigenome Project (<http://www.epigenome.org/>). Mapping the human epigenome—the epigenetic variability in cells—holds the hope for precise diagnosis of disease and congenital anomalies. While multiple molecular mechanisms underlie these processes, the most actively investigated to date include DNA methylation, chromatin remodeling and the action of miRNAs.

In the 1960s, a gene was defined simply as that specific stretch of DNA containing the instructions to make a protein. Complications to our comfort level of understanding emerged in the 1980s and 1990s when it was determined that vast stretches of noncoding DNA lie between the coding exonic regions. The 22,000 or so protein coding genes in the human genome make up just 1.2% of that genome. Indeed, in 2000, the first rough draft of that genome left the other 98.8% of the human genome largely unexplored. Since then, fine mapping (identification of markers that are tightly linked to a targeted gene) and deep sequencing contributing to the encyclopedia of DNA elements (encode) suggest that a staggering 93% of the genome produces RNA transcripts. MicroRNAs represent the largest family of noncoding RNAs involved in gene silencing (Conrad et al., 2006; Lee et al., 2006), and are critical to cell and tissue differentiation (Mineno et al., 2006; O'Rourke et al., 2006; Yang and Wu, 2006). miRNAs regulate nearly 30% of protein coding genes, rivaling transcription factors in importance as orchestrators of gene expression. The number of transcription factors binding at different sites varies, due to SNPs and structural variation near different genes, influencing the level of gene expression (Kasowski et al., 2010). Much as methylation (see below), variation of transcription factor binding acts both as a switch and a dial-determining which genes are turned on or off, and how much message is produced by an “on” gene. Thus, transcription factors, which account for as much as 10% of the coding genome in humans, and miRNAs, represent the key regulators of gene expression and cell/tissue differentiation.

MicroRNAs regulate expression of genes post-transcriptionally by binding to, and then inhibiting the translation of, and/or destabilizing their target mRNAs (Bartel, 2009). Their role in development of the secondary palate has recently begun to emerge. An increasing number of miRNAs have been shown to target many of the signaling mediators discussed above as being important effectors of palatal morphogenesis (Kawasaki and Taira, 2003; Li and Carthew, 2005; Martello et al., 2007; Kennel et al., 2008; Zhao et al., 2008; Lin et al., 2009). For example, TGF β -mediated epithelial-mesenchymal transformation (EMT) (Sun et al., 1998a, b) was shown to be associated with marked downregulation of *miR-205* and all five members of the microRNA-200 family (*miR-200a*, *miR-200b*, *miR-200c*, *miR-141*, and *miR-429*) (Gregory et al., 2008). Importantly, induced expression of the miR-200 family alone was sufficient to prevent TGF β -induced EMT. Moreover, in zebrafish, microRNA 140 (*Mir140*) was shown to inhibit platelet-derived growth factor receptor α -mediated attraction of cranial neural crest cells to the oral ectoderm (Eberhart et al., 2008), a process essential for normal morphogenesis of the secondary palate.

Determination of miRNA expression patterns, while criticized by some as being “simply” descriptive, are particularly important because expression of avian (Darnell et al., 2006; Xu et al., 2006), zebrafish (Giraldez et al., 2005), and murine (Takada et al., 2006) miRNAs are tightly controlled in a tissue- and developmental stage-specific manner during organogenesis (Plasterk, 2006; Saetrom et al., 2007). In a recent study, unique signatures of hundreds of miRNAs expressed during embryonic orofacial development were defined (Mukhopadhyay et al., in press). Further, miRNAs within developmentally related clusters were shown to target a panoply of genes encoding proteins involved in ECM metabolism, cell proliferation, cell adhesion, cell differentiation, apoptosis and epithelial-mesenchymal transformation, all

processes critical for normal orofacial development. As discussed by these authors (Mukhopadhyay et al., in press), several differentially expressed miRNAs, including *Mir-193*, members of the let-7 family of miRNAs, *Mir-140*, *Mir-122*, and *Mir-152* were identified as potentially central regulators of downstream mRNAs encoding proteins known to play pivotal roles in orofacial development. Moreover, during development of palatal tissue, differentially expressed miRNAs also were shown to target cytoskeletal proteins, growth and differentiation factors, signal transduction modulators and effectors, and transcription factors—all having been shown (see above) to play crucial roles in orofacial ontogeny. To determine how differentially expressed miRNAs and their target genes might interact in the developing murine orofacial tissue, an Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) was performed (Mukhopadhyay et al., in press). One representative network with eight differentially expressed miRNAs - *mir-20a*, *mir-20b*, *mir-22*, *mir-106a*, *mir-140*, *mir-206*, *mir152*, and *mir-362*, is shown in Figure 8. Interestingly, this gene network contains several direct and indirect target genes (of the differentially expressed miRNAs), known to be expressed in embryonic orofacial tissue (*Cux1*, *Foxc1*, and *Mybl1*) (Mukhopadhyay et al., 2006a, b), or to execute crucial roles during orofacial ontogenesis: *CREBBP* (Warner et al., 2004), *CBP/p300* (Warner et al., 2006), and *CDK* (Dhulipala et al., 2006). Overlay of this miRNA-mRNA gene network with relevant biological functions highlighted numerous cellular processes crucial for orofacial growth and maturation (see Fig. 9). Such gene networks clearly document the paramount significance of the various developmentally regulated miRNAs in directing expression of key genes orchestrating orofacial ontogenesis. Collectively, the data support the notion that differentially expressed miRNAs, regulating crosstalk among diverse signal transduction pathways, govern tissue differentiation and morphogenesis of developing orofacial tissue.

Finally, to demonstrate that the differentially expressed miRNAs are directly linked to potential target genes known to play a critical role in orofacial development, two additional gene networks were generated. One network, using miRNAs exhibiting enhanced expression (see Fig. 10) (Mukhopadhyay et al., in press), and the other utilizing miRNAs exhibiting diminished expression (not shown) in embryonic orofacial tissue, were developed utilizing IPA and the miRDB online database (<http://mirdb.org/miRDB/>). It should be noted that virtually all of the target genes of upregulated miRNAs (see Fig. 10) have been shown to play a role in orofacial development. Examples include, but are not limited to, the *TGFβs* and *Bmps* (see above), *Meox-2* (Jin and Ding, 2006b; Li and Ding, 2007), *PDGF* (Xu et al., 2005; Choi et al., 2009a, b), *MMPs* (Blavier et al., 2001; Brown et al., 2002; Letra et al., 2007; de Oliveira Demarchi et al., 2010), *Pitx2* (Szeto et al., 1999), *Fgfr1* (Lee et al., 2008), *Satb2* (Leoyklang et al., 2007), and *Dlx5* (Han et al., 2009).

EPIGENETICS: METHYLATION

Methylation of DNA is one of the most common epigenetic modifications regulating gene expression in mammalian cells (Shiota, 2004). This involves transfer of a methyl group from *S*-adenosyl methionine to carbon-5 of a cytosine ring, introducing 5-methylcytosine as a new base on DNA (see Fig. 11). Generally, methylation occurs at cytosines that are contained in a symmetrical CpG dinucleotide sequence (Jones and Takai, 2001). These sequences—approximately 30,000 in number in the genome—tend to concentrate in regions known as CpG islands, containing a G-C content of $\geq 55\%$, and are located in the promoter regions of genes. These islands are excellent substrates for DNA methyltransferases (Antequera, 2003). Indeed, mRNA microarray analysis of total RNA extracted from gd 9.5 murine embryonic craniofacial tissue, demonstrate the expression of many methyltransferases and methyl CpG binding proteins in the developing embryonic craniofacial region (Table 2). This provides evidence that tissue comprising the early developing embryonic orofacial region is able to carry out DNA methylation.

CpG methylation of CpG islands in gene promoters correlates with transcriptional silencing (Antequera, 2003; Caiafa and Zampieri, 2005) via inhibition of transcription factor binding (Geiman and Robertson, 2002), or interaction of methyl CpG binding proteins with transcriptional repressors (Ohashi et al., 2004). DNA methylation patterns during embryogenesis are nonrandom and contribute to tissue-specific gene expression (Monk et al., 1987; Kafri et al., 1992). Indeed, nearly 50% of CpG islands are linked to tissue-specific genes (Suzuki et al., 2001), and the methylation pattern of CpG islands is unique in each tissue or cell type (Shiota et al., 2002). Failure to establish correct methylation patterns can lead to embryonic lethality (Li et al., 1992), or can result in developmental craniofacial malformations (Matsuda and Yasutomi, 1992; Kakutani et al., 1996; Ohgane et al., 2001; Gonzales et al., 2005; Abu-Amro et al., 2008), including cleft palate (Blik et al., 2008; Kuriyama et al., 2008; Loenarz et al., 2010).

Environmental factors such as alcohol use, exposure to cigarette smoke, drugs, and chemicals (e.g., pesticides), as well as altered intake of nutrients (e.g., trace elements and folate) have been implicated in the etiology of orofacial malformations (Honein et al., 2007; Romitti et al., 2007; Grewal et al., 2008). Such environmental insults could trigger aberrant DNA methylation in susceptible genes during embryonic or fetal development. Importantly, such alterations cannot be detected by conventional screening techniques such as mutation screening or Genome-Wide Association Analysis (GWAS). Whereas in the past, genetic predisposition to craniofacial defects was—and continues to be—sought among polymorphisms (RFLPs and SNPs), deletions, or copy number variations, we can now begin to think about miRNA expression profiles and downstream promoter methylation sequelae as possible risk factors for craniofacial defects. The gene encoding the transcription factor *Sox4* serves as an instructive example.

Sox4 is widely expressed in the murine embryo (Dy et al., 2008) and is thought to represent a key player in regulating multiple aspects of neural crest cell development (Hong and Saint-Jeannet, 2005). In addition, *Sox4* has recently been identified as a strong causative candidate gene for human nonsyndromic cleft lip and palate (CLP) (Juriloff and Harris, 2008). Gene expression analysis of murine embryonic palatal tissue revealed significant temporal downregulation of the *Sox4* gene (Mukhopadhyay et al., 2006a, b). Since CpG methylation of upstream regulatory elements is typically associated with repression of gene expression resulting in reduced mRNA levels, the CpG methylation profile of *Sox4* was determined (Seelan, preliminary data) using genomic DNA isolated from the developing murine palate (Clark et al., 1994). Amplicons from the upstream region of *Sox4* were cloned into pGEM-T Easy and sequenced to determine the methylation levels of all resident CpG residues. Comparison of percent methylation of amplicons 1.8 kb upstream of the ATG start site of *Sox4* revealed that significant methylation differences were seen only in two amplicons of the *Sox4* promoter, and only on gestational day 12 (see Fig. 12). These represent “Differentially Methylated Regions” (DMRs) for *Sox4*. The CpG island of the *Sox4* promoter, represented by amplicon 1 (see Fig. 12), is relatively unmethylated. While challenging the generally held notion that the methylation status of promoter CpG islands is a key factor in regulation of gene expression, the data are consistent with recent studies indicating that a majority of methylation changes (>94%) do not occur in CpG islands, as previously thought, but in upstream regions called “CpG island shores” (Irizarry et al., 2009). Because targets of *Sox4* include 23 other transcription factors, as well as numerous key cellular regulators, including those of both the TGF β and Wnt- β -catenin signaling pathways (Scharer et al., 2009), aberrant expression of this gene via epigenetic mechanisms, may have profound and deleterious developmental effects on systems—such as the craniofacial complex—dependent on these pathways for normal ontogeny.

Galileo wrote that “...Nature’s great book is written in the language of mathematics.” Genomic array technology, utilizing mathematical algorithms to illuminate gene expression and methylation profiles, as well as pathways critical for morphogenesis—as described above for miRNA and methylation analyses—has provided fascinating insights into the development of a spectrum of biological systems including the developing orofacial region. Because of such approaches, and despite the fact that such analyses are statistical and not deterministic, the cast of gene and protein characters that play on the cellular stage of orofacial ontogeny continues to expand (Karoly et al., 2005; Mukhopadhyay et al., 2006a, b, in press).

LOOKING BACKWARD AND LOOKING FORWARD

Someone once observed how foolish medieval Europeans, living before the time of Copernicus, must have been that when looking at the sky, they thought that the sun was circling the earth. Surely a modicum of astronomical good sense would have told them that the reverse was true. . . . yet . . . what would it have looked like if the sun *had* been circling the earth? The answer is that it would look exactly the same! The point of this is, that when we observe Nature, what we see is shaped according to what we believe we know about it at the time. As indicated in the abstract of this review, our objective herein was to focus on a discussion of the “facts” of palatal ontogeny as they relate to cellular signal transduction. The current overview represents a construct, albeit limited, of our collective database and interpretation of its meaning, based on what we think, for the present, are the facts.

“There is no place for dogma in science. The scientist is free, and must be free to ask any question, to doubt any assertion, to seek any evidence, to correct any error.”

J. Robert Oppenheimer

1904–1967

For the past 50 years, scientists have pursued fundamental knowledge about the developing embryo and application of that knowledge to reduce the burden of disabilities. The current mantra for contemporary research addressing the causes of congenital malformations is one that strives for “bench to bassinet” outcomes. Biomedical advances are increasingly being made by research teams, using interdisciplinary approaches and supported by open access to powerful databases and online tools. Indeed, many investigators are finding that their most powerful research tool has become the computer (see article by Knudsen in this issue). Despite the use of such approaches (see above), many of the underlying molecular events responsible for orofacial morphogenesis persist in eluding our complete understanding. While much has been learned, heterogeneity among orofacial cleft defects, as well as complexity of inheritance, has made elucidation of underlying causes difficult.

Over 200 years ago, the French naturalist Jean-Baptiste Lamarck proposed the notion that acquired characteristics can be passed on to offspring. In the shadow of the Darwinian/Mendelian dictum that organisms are strictly determined by their genetic make-up, essentially isolated from the environment, the Lamarckian inheritance of acquired characteristics was considered little more than an interesting historical sidebar with little scientific validity. Current biological thought recognizes that epigenetics represents a fundamental contributing process in embryogenesis, and that the environment can have a profound effect on shaping the epigenome. The most notable evidence comes from seminal studies utilizing agouti mice. These studies dramatically demonstrate that diet can result in heritable phenotypic change—in a single generation—not by changing DNA sequence but by changing the DNA methylation pattern of the genome (Waterland and Jirtle, 2003;

Dolinoy and Jirtle, 2008). These, and an increasing number of other studies which reach similar conclusions, provide rationale for reconsideration of the long-refuted notions about the inheritance of acquired characteristics.

“Every great scientific truth goes through three stages:

First, people say it conflicts with the Bible.

Next they say it had been discovered before.

Lastly, they say they always believed it.”

Jean Louis Agassiz (1807–1883)

While methylation of DNA is a common epigenetic modification that contributes to the control of gene expression in mammalian cells, the notion of tissue/cell-specific methylation as a means of regulating morphogenesis of the orofacial region is gaining currency. Epigenetic changes, such as methylation, are reversible and thus present attractive therapeutic targets. Indeed, “epigenetic drugs” such as inhibitors of DNA methyltransferases or/and histone deacetylases can reverse epigenetic marks on DNA and histones resulting in dramatic alterations in gene expression (Hamm et al., 2009; Li et al., 2009). This potential to epigenetically reverse nuclear methylation and/or acetylation to re-express affected critical genes presents an attractive future option for exploring clinical use in cases where epigenetically induced gene silencing or overexpression contributes to abnormal developmental outcomes such as the induction of orofacial clefts. In this regard, since imprinted genes are expressed from only one of the two parental alleles, it is significant that Prader-Willi (Xin et al., 2003), Angelman’s (Dan, 2009; Gurrieri and Accadia, 2009), and Beckwith-Wiedemann (Higashimoto et al., 2003; Gurrieri and Accadia, 2009) syndromes, as well as immunodeficiency centromere instability (ICF) syndrome with its attendant facial abnormalities (Ehrlich, 2003), all result from abnormal demethylation of the silenced allele of imprinted genes causing biallelic expression.

The remarkable successes of biomedical research notwithstanding, the frequency of congenital craniofacial anomalies remains distressingly high. Despite an impressive broadening of our understanding of the signaling cascades orchestrating orofacial ontogeny—some of which have been outlined herein—the fundamental mechanisms governing these processes in normal orofacial development, and their dysregulation resulting in orofacial clefting, remain poorly defined. When depicting far off lands at the edge of the known world, maps from the Middle Ages declared “Here There Be Dragons.” While our understanding of the molecular mechanisms that orchestrate orofacial development has dramatically increased in the past several decades, our knowledge remains uncomplicated by a full appreciation of the regulatory intricacies involved in normal, much less abnormal, development of the orofacial complex. Here there be dragons indeed!

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Figure 1.

Development of the midfacial primordia. Scanning electron micrographs of the developing orofacial region showing the prominences that give rise to the main structures of the face. (A) mouse gestational day (GD) 10, equivalent to human 5th week of development, (B) mouse GD 11, equivalent to human 6th week of development, and (C) human 6th week of development. The mandible is formed by merging of the homologous mandibular processes (MP) of the first branchial arch. The upper lip is formed by merging of the bilateral maxillary processes (MX) of the first branchial arch with the medial nasal processes (MNP), which merge with each other. The lateral nasal processes (LNP) give rise to the alae, or sides, of the nose. Reprinted with the permission of Dr. Kathleen Sulik, University of North Carolina, Chapel Hill, N.C.

Development of Secondary Palate

Figure 2.

Mammalian palate development. Adapted from Langman's Medical Embryology (Sixth Edition) with permission from the publisher, Williams and Wilkins.

Figure 3.

Enhanced green fluorescent protein (EGFP)-labeled neural crest cells in *Wnt1-Cre/Z-EG* transgenic mouse embryos. Photomicrograph of a gestational day (GD) 9.5 two-component *Wnt1-Cre/Z-EG* transgenic mouse embryo under epifluorescence optics showing EGFP expression in the developing orofacial region in the neural crest cells of the first and second branchial arches (BA1 and BA2) and in the neural crest cells of the frontonasal region (FN) marco-RAFT agents.

Figure 4.

Coronal paraffin sections of the developing murine orofacial region on day 14 of gestation. By murine gestation day 14, individual palatal processes (P) (A) have made contact, thereby separating the oral cavity (below the palate), from the nasal cavity (above the palate). Nasal cavity (N), tongue (T), Meckel's cartilage (*arrows*), are of medial edge epithelial seam (circled). (B) Higher magnification of a region similar to that circled in (A), demonstrating that immunostaining for TGF β 2 (arrow) predominates in the epithelium of adjacent palatal processes. Adapted from Gehris et al., *Int J Dev Biol* 1991;35:1–8. marco-RAFT agents.

Figure 5.

Transcriptional activation of a BMP-inducible luciferase reporter plasmid in murine embryonic maxillary mesenchymal (MEMM) cells. Primary cultures of MEMM cells were co-transfected with 1.0 μ g of a BMP-responsive plasmid BRE-luc and 0.1 μ g of the control plasmid pRL-CMV for 24 hr, followed by 24 hr exposure to either vehicle, 2 ng/ml TGF β 1, or 25, 50, 100 ng/ml BMP2 or BMP4. Cells were extracted, luciferase activities were measured, and firefly luciferase activity was expressed relative to Renilla luciferase activity to normalize for transfection efficiency. Mean luciferase activities and standard errors were calculated from triplicate cell culture samples, and treatments compared by analysis of variance (ANOVA), followed by Student's *t* test. *p* values <0.05 were considered statistically significant. *Indicates luciferase activity significantly different from control. Adapted from Mukhopadhyay et al., *Birth Defects Res A* 2006;76:528–543.

Figure 6.

Progression of the craniofacial phenotype after Shh inhibition. Forty-eight hours after treatment (A, C), control embryos reach St. 18–20. There are no indications of gross morphological disruptions to the craniofacial complex. The eyes (ey) are prominent and the retina is pigmented. The telencephalon (te) is divided into right and left halves, and the maxillary (mx), mandibular (ma), and hyoid (hy) arches are apparent, and the nasal pits (np) are widely spaced (dotted bracket). (B, D) Treated embryos also reach St. 18–20 by 48 hr after the injection of 5E1 cells; embryos exhibit marked morphological alterations of the craniofacial complex. The eyes are reduced in size. The telencephalon is divided into right and left halves, but they appear smaller than the controls. The nasal pits are present and deepening, but they are close together (bracket). The maxillary processes converge toward the mid-line and the eyes are rotating ventrally, while the mandibular and hyoid arches are unaffected. (D) In the lateral view, the craniofacial complex in treated embryos appears blunt. The entire frontonasal and maxillary primordia appear shorter than (C) the controls. Within 72 hr of treatment, normal, (E, G) control, and (F, H) treated embryos reach St. 20–24. (E) In control embryos, the eyes have enlarged and the facial primordia are well defined. (F) By 72 hr, the morphological changes present at 48 hr have become more pronounced. (G) Lateral view of the control embryo showing that the maxillary process extends past the anterior edge of the eye. (H) Lateral view of treated embryo indicating that the maxillary process extends beyond the anterior edge of the eye, but the eye is reduced in size. The entire frontonasal primordium appears blunted compared with control embryos. di, diencephalon; me, mesencephalon. Scale bars: 0.5 mm. Reprinted with the permission of the author, Dr. Ralph Marcucio, University of California at San Francisco, San Francisco, CA, and Elsevier Publishing. Adapted from Marcucio et al., *Dev Biol* 2005;284:48–61.

Figure 7.

Wnt-2 expression in mesenchyme of the developing secondary palate. Cryo-sections of fixed, frozen heads from mouse gestation days (GD) 12.5–14.5 were processed for expression of Wnt-2 transcripts by in situ hybridization with either an antisense Wnt-2-specific riboprobe (A–C) or the sense (negative) control probe (D–F). No detectable expression of Wnt-2 was observed in GD 12.5 palates (A, palatal shelf outlined in black). Wnt-2 transcripts were, however, detected in the mesenchyme subjacent to the epithelium forming the junction between the oral cavity and tongue (*arrows*). On GD 13.5 and 14.5, expression of Wnt-2 was detected in the mesenchyme of the palatal shelf (B and C, *arrows*) and extending laterally into the maxilla. No signal was obtained with a sense Wnt-2 riboprobe (D–F). (A, B, D, E) $\times 200$ magnification; (C, F) $\times 100$ magnification. Size bar is 50 μm . Whole-mount in situ hybridization was also performed (G–M). No Wnt-2 mRNA was detected on GD 12.5 (G), but on GD 13.5 (H) a domain lateral to the medial edge of the palatal shelves and restricted to the anterior one-third of the palate was observed (*black arrows*). On GD 14.5, the Wnt-2 mRNA expression domain was still confined to the anterior palate with signals also observed in the nasal septum (M, *arrows*). Because the signal on GD 14.5 samples was difficult to discern on darkfield images (I), a brightfield image is included (M) that clearly demonstrates Wnt-2 expression using the antisense riboprobe. No signals were obtained when a Wnt-2 sense riboprobe was used (J–L and M). UL, upper lip; PP, primary palate; PS, palatal shelf; NS, nasal septum; MES, medial edge seam. The fused secondary palate is outlined in (I). Adapted from Warner et al., *Int J Devel Biol* 2009;53:1105–1115. marco-RAFT agents.

Figure 8.

Computational gene interaction predictions from a selected microRNA gene network in murine orofacial development. Several differentially regulated microRNA genes in developing murine orofacial tissue (shown in aqua) were used to construct a gene association map using Ingenuity Systems Pathway Analysis (IPA) software. Solid lines specify direct relationships between genes whereas dotted lines indicate indirect interactions.

Figure 9.

Computational gene interaction and biological function predictions from a selected microRNA gene network in murine orofacial development. Several differentially regulated microRNA genes in developing murine orofacial tissue (shown in aqua) were used to construct a gene association map (shown in Fig. 8) using Ingenuity Systems Pathway Analysis (IPA) software to predict various cellular and molecular events regulated by these microRNAs (shown in this figure) during orofacial development. Solid lines specify direct relationships between genes whereas dotted lines indicate indirect interactions.

Figure 10.

Computational gene interaction predictions: gene network with microRNAs demonstrating enhanced expression in developing orofacial tissue (GD-13 vs. GD-12) and their target genes. A network with selected genes encoding microRNAs (orange) that demonstrate increased expression between GD-12 and GD-13 of orofacial development, and their known or predicted target genes (yellow) critical for orofacial ontogenesis was constructed with Ingenuity Systems Pathway Analysis (IPA) software and the miRDB (<http://mirdb.org/miRDB/>) database. Solid lines specify direct relationships between genes whereas dotted lines indicate indirect interactions.

Figure 11.

DNA methylation. DNA methylation involves the covalent addition of a methyl group from *S*-adenosyl methionine (SAM), catalyzed by DNA methyltransferases, to the 5-carbon of cytosine in a CpG dinucleotide.

Figure 12.

CpG methylation profile of the *Sox4* gene upstream regulatory region during murine secondary palate development. An 1.7 kb upstream region of the *Sox 4* gene, beginning from the ATG start site (shown on the right), is defined by eight amplicons (boxed). Individual CpG residues are numbered within the boxes. Percentage methylation—the average of all methylated CpG residues within an amplicon—is shown below each amplicon for murine gestation day (GD) 12, GD 13, and GD 14 secondary palate. *Yellow boxes* represent differentially methylated regions (DMRs); *blue boxes* represent unmethylated regions; and *red boxes* represent highly methylated regions. Amplicon 2 was not analyzed as it was presumed to be unmethylated based on analysis of amplicons 1 and 3. Amplicon 6 could not be amplified. The CpG island, an area of high CpG density is located in the area of amplicon #1.

TABLE 1

Genes Encoding Members of the TGF β Superfamily and Associated Signaling Proteins That Are Differentially Expressed During Murine Orofacial Development^{a,b}

Gene descriptor	Relative change	
	GD 13 vs. GD 12 ^c	GD 14 vs. GD 13 ^c
TGF β 1	-1.12	6.50
Latent TGF β binding protein 1	1.10	2.14
Calpain 10	1.05	-5.04
Retinoid X receptor gamma	-1.05	2.10
Frat1	-2.20	-1.20
Prostaglandin F receptor	2.83	1.07
Decorin	3.33	1.62
Dachshund 1	1.35	-2.30
BMP 10	-2.52	1.63
BMP 15 or GDF 9B	1.41	-2.70
Protease, serine, 11	2.20	2.25
BMP receptor, type II	-2.14	1.18
Cerberus 1	3.18	-2.14
Inhibin alpha	2.25	-1.55
Thrombopoietin	-2.41	1.18
Sox4	-2.05	-1.02
Stat4	2.56	-2.00
Cyclin-dependent kinase inhibitor kip1 (p27)	-1.91	-1.15
Cyclin-dependent kinase inhibitor 1A (p21)	1.78	2.20
Cyclin dependent kinase inhibitor 2B (p15)	3.18	1.18
Alpha 1 type I procollagen	1.80	3.03
Fos (FBJ osteosarcoma oncogene)	-3.33	1.50
Involucrin	1.55	-2.10
Interleukin 6	2.50	-1.45
MMP2 (gelatinase A)	-2.41	2.35
MMP9 (gelatinase B)	6.96	1.12

^aWhole genome transcription profiling of murine orofacial tissue from gestation day (GD) 12, GD 13, and GD 14 was conducted utilizing oligonucleotide-based high-density Affymetrix GeneChip arrays.

^bModified from Mukhopadhyay et al., Birth Defects Res A 2006;76:528–543.

^cTriplicate gene expression data sets from fetal orofacial tissue from gestation day (GD) 12, GD 13, and GD 14 were filtered and the relative change in expression for each gene was calculated following comparisons of the gene expression in fetal orofacial tissue on GD 13 versus GD 12 and GD 14 versus GD 13. Only those genes that demonstrated a statistically significant ($p < 0.005$) increase or decrease in expression of at least twofold in all three replicates are included. For GD 13 versus GD 12 comparisons, expression on day 12 was utilized as the baseline, and for GD 14 versus GD 13 comparisons expression on day 13 was utilized as the baseline. Therefore, negative numbers indicate a relative decrease in expression, whereas positive numbers indicate a relative increase in expression.

TABLE 2DNA Methylation Genes Expressed in the Developing Murine Orofacial Region^a

DNA methyltransferases	Methyl-CpG binding domain proteins
<i>Dnmt1</i>	<i>Mbd1</i>
<i>Dnmt2</i>	<i>Mbd2</i>
<i>Dnmt3a</i>	<i>Mbd3</i>
<i>Dnmt3b</i>	<i>Mbd4</i>
	<i>MeCP2</i>

^aWhole genome transcription profiling of first branchial arch tissue from gestation day 9.5 mouse embryos was conducted utilizing oligonucleotide-based high-density Affymetrix GeneChip arrays revealing expression of the above genes encoding methyltransferases and methyl-CpG binding domain proteins. Gene expression in the tissue was subsequently verified by Taq-Man real-time PCR.