Developmental Epigenetics of the Murine Secondary Palate

Ratnam S. Seelan, Partha Mukhopadhyay, M. Michele Pisano, and Robert M. Greene

Abstract

Orofacial clefts occur with a frequency of 1 to 2 per 1000 live births. Cleft palate, which accounts for 30% of orofacial clefts, is caused by the failure of the secondary palatal processes—medially directed, oral projections of the paired embryonic maxillary processes—to fuse. Both gene mutations and environmental effects contribute to the complex etiology of this disorder. Although much progress has been made in identifying genes whose mutations are associated with cleft palate, little is known about the mechanisms by which the environment adversely influences gene expression during secondary palate development. An increasing body of evidence, however, implicates epigenetic processes as playing a role in adversely influencing orofacial development. Epigenetics refers to inherited changes in phenotype or gene expression caused by processes other than changes in the underlying DNA sequence. Such processes include, but are not limited to, DNA methylation, microRNA effects, and histone modifications that alter chromatin conformation. In this review, we describe our current understanding of the possible role epigenetics may play during development of the secondary palate. Specifically, we present the salient features of the embryonic palatal methylome and profile the expression of numerous microRNAs that regulate protein-encoding genes crucial to normal orofacial ontogeny.

Key Words: cleft palate; CpG islands; DMR; methylation promoter arrays; methylome; microRNAs; secondary palate

Introduction

Development of the secondary palate serves as a paradigm for embryonic development in general, inasmuch as virtually all of the molecular processes and signaling pathways associated with normal palatal ontogeny are mirrored in the embryogenesis of multiple other systems. The secondary palate (roof of the mouth) separates the oral cavity from the nasal cavity. A defect in the development of the secondary palate causes cleft palate (CP[1]), which, if left untreated, can lead to feeding and speech impediments, hearing loss (and ear infections), and breathing difficulties (MacLean et al. 2009; Nackashi et al. 2002; Wehby and Cassell 2010). Orofacial clefts occur with a frequency of 1 to 2 per 1000 live births and represent half of all craniofacial anomalies (Stanier and Moore 2004). Besides CP, orofacial clefts also include cleft lip with or without cleft palate (CL/P[1]), a disorder attributed primarily to defects in the formation of the upper lip. CP constitutes approximately 30% of orofacial clefts and occurs either as an isolated defect (nonsyndromic) or as a component of a syndrome, of which there are more than 200 that include CP as part of their phenotype.[2] Some common syndromes that manifest CP include Apert's (Ibrahimi et al. 2005), Stickler's (Johnson et al. 2011), and Treacher Collins (Trainor 2010), whereas CL/P is primarily associated with Van der Woude's (Rizos and Spyropoulos 2004) and Patau's (Patau et al. 1960) syndromes. Population-based studies, as well as our current understanding of the molecular underpinnings of orofacial development, suggest that gene mutations responsible for CL/P and CP are distinct (Fogh-Anderson 1942; Juriloff and Harris 2008; Murray 2002; Spritz 2001).

Both gene mutations and environmental effects underlie the complex etiology of CP (Beaty et al. 2011; Fraser and Calnan 1961; Greene and Pisano 2010). Although some progress has been made in identifying genes that, when mutated, cause CP, very little progress has been made in elucidating how environmental factors contribute to this defect. Environmental influences can adversely affect development of the secondary palate by means of genetic variants or epigenetic alterations. Single-nucleotide polymorphisms (SNPs[1]) in specific genes can confer an increased risk to

Ratnam S. Seelan, PhD, and Partha Mukhopadhyay, PhD, are assistant professors in the Department of Molecular, Cellular and Craniofacial Biology, Birth Defects Center; M. Michele Pisano, PhD, is a professor in the Department of Molecular, Cellular and Craniofacial Biology and director of the Birth Defects Center and NIH Center of Biomedical Research Excellence (COBRE); and Robert M. Greene, PhD, is professor and chair of the Department of Molecular, Cellular & Craniofacial Biology and director of the Birth Defects Center and NIH Center of Biomedical Research Excellence (COBRE), all at ULSD, University of Louisville, Kentucky.

Address correspondence and reprint requests to M. Michele Pisano, Department of Molecular, Cellular and Craniofacial Biology, Birth Defects Center, ULSD, University of Louisville, 501 S. Preston Street, Louisville, KY 40202 or email pisano@louisville.edu.

[1]Abbreviations that appear ≥3x throughout this article: BMP, bone morphogenetic protein; cAMP, cyclic adenosine monophosphate; CP, cleft palate; CL/P, cleft lip with or without cleft palate; DMR, differentially methylated region; ECM, extracellular matrix; EMT, epithelial mesenchymal transition; GD, gestational day; miRNA, microRNA; mRNA, messenger RNA; PDGF, platelet-derived growth factor; SHH, Sonic hedgehog; SNP, single-nucleotide polymorphism; TGF, transforming growth factor; WNT, Wingless-type MMTV integration site.

[2]Data from Online Mendelian Inheritence in Man, www.ncbi.nlm.nih.gov/omim (this and other websites cited in this article were accessed on November 5, 2012).

adverse developmental outcomes subsequent to exposure to certain environmental hazards. For instance, infants carrying a specific transforming growth factor alpha (TGFα[1]) polymorphism and whose mothers smoked cigarettes during their pregnancy exhibit a significantly increased risk for CP (Beaty et al. 1997; Hwang et al. 1995; Shaw et al. 1996). Although SNPs are easily identifiable because they involve a change in DNA sequence, epigenetic alterations can trigger gene expression changes without altering DNA sequence. Consequently, epigenetic changes cannot be identified by techniques such as genome-wide association studies that scan DNA for nucleotide changes. Epigenetic mechanisms include, but are not limited to, DNA methylation, microRNA (miRNA[1]) function, and histone modification. In this review, we focus on the current state of epigenetics as it relates specifically to (murine) secondary palate development and to processes that contribute to CP.

Murine Secondary Palate Development

Morphogenesis and Signaling Pathways

Orofacial development primarily begins with the migration of neural crest cells derived from the neuroectoderm of rhombomeres 1 to 3 (Köntges and Lumsden 1996) into the first two branchial arches, followed by the diversification of neural crest cell fates (Jheon and Schneider 2009; LaBonne and Bronner-Fraser 1999). Growth of the secondary palate depends on the survival of, synthesis of extracellular matrix (ECM[1]) by, and active proliferation of mesenchymal cells derived from the cranial neural crest and mesodermal cells of the first branchial arch. The palatal processes (shelves) originate as bilateral extensions of the oral aspect of the maxillary processes and eventually fuse to give rise to the secondary palate. In mice, these events occur during gestational days (GDs[1]) 12 to 14 and during the 7th week of gestation in humans (Figure 1). Elevation of the paired palatal processes and their subsequent adhesion and fusion are highly conserved in mammals. Processes that affect the size of the palatal processes, their reorientation to a position above the tongue, or their fusion to each other can contribute to CP. Developmental processes that underlie these morphogenetic events include cell proliferation, ECM metabolism, epithelial mesenchymal transition (EMT[1]), apoptosis, cell migration, and the activity of a diverse array of signal transduction pathways. Mutation analyses in mice indicate that many of the genes responsible for development of the secondary palate encode transcription factors, growth and signaling molecules and their receptors, and ECM components (Carinci et al. 2007; Cobourne 2004; Gritli-Linde 2007; Jugessur et al. 2009; Juriloff and Harris 2008; Lidral and Moreno 2005; Murray and Schutte 2004; Murthy and Bhaskar 2009; Rice 2005; Stanier and Moore 2004; Vieira 2008; Yu et al. 2009). Several of the genes that play essential roles in palate development are members of key signal transduction pathways, including the wingless-type MMTV integration site (WNT)-, TGFβ-, platelet derived growth factor (PDGF)-, fibroblast growth factor–, and Sonic hedgehog (SHH)–signaling systems (Greene and Pisano 2010; Gritli-Linde 2007; Jugessur et al. 2009; Murray and Schutte 2004; Yu et al. 2009).

In this review, we describe results from our published and ongoing studies that examine two types of epigenetic mechanisms—changes in DNA methylation and regulation of gene expression by miRNAs—during development of the murine secondary palate.

Epigenetics and the Secondary Palate

Epigenetics is the study of inherited changes in phenotype or gene expression by means of mechanisms that do not alter the DNA sequence. The most actively investigated epigenetic mechanisms are DNA methylation of C in CG (often referred to as CpG) dinucleotides, the action of miRNAs, and chromatin remodeling involving histone modifications. Aberrant DNA methylation of susceptible genes can result in gene silencing, changes in miRNA levels can affect transcription of target genes, and alterations in histone methylation can affect chromatin conformation (closed and open conformations repress and facilitate gene transcription, respectively). Such epigenetic alterations may adversely affect key biochemical pathways and developmental processes that are

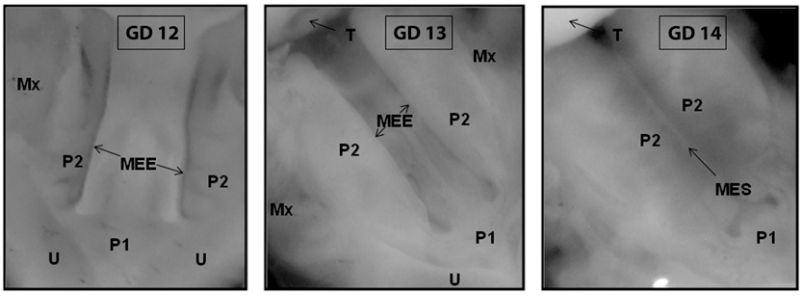

Figure 1 Key stages in the development of the mammalian secondary palate. Panels depict ventral views of the oral cavity with the tongue removed. Formation of the secondary palate occurs between gestational day (GD) 12 and GD 14 in mice (shown) and gestational weeks 6.5 to 10 in humans, in which the developmental steps depicted are similar. During this process, the pair of secondary palatal shelves (P2) grow toward each other, make contact along their respective medial edge epithelia (MEE; GD 12 and GD 13) to form the medial edge seam (MES; GD 14), effectively separating the oral from the nasal cavities. Mx, maxilla; P1, primary palate; P2, secondary palate; T, tongue; U, upper lip.

temporally programmed and requisite for normal palatal development. These epigenetic mechanisms are not mutually exclusive. For instance, a decrease in miRNA levels could be due to DNA methylation of regulatory regions/CpG islands of miRNA-encoding genes (Chen et al. 2012; Vrba et al. 2010). Methylation-induced gene silencing can occur either by impeding the binding of transcription factors and/or the methyl group serving as a substrate for methyl-CpG binding proteins (Bogdanović and Veenstra 2009). The latter may, in turn, recruit chromatin remodeling proteins that affect various histone modifications to establish a repressive (condensed) chromatin environment that results in dramatically altered levels of gene expression (Deaton and Bird 2011). Gender differences are also observed in clefting phenotypes (Derijcke et al. 1996; James 2000; Tolarova 1990). It is noteworthy that isolated CP occurs twice as frequently in females as in males, and paternal, but not maternal, age is strongly associated with CP (Bille et al. 2005). At least one gene, *TBX2*, which is located on the X chromosome, has been found to be mutated in patients with CP (Andreou et al. 2007). Although it is not clear if these effects are all sex chromosome–based, it is possible that they could be due to differential methylation of specific autosomal genes in males and females. Interestingly, several sexually dimorphic genes have been observed to occur in liver, adipose tissue, muscle, and brain, which implies that gene expression differences between sexes are prevalent in many somatic tissues (Yang et al. 2006).

Indications that gene methylation might contribute to the etiology of CP first began to appear in the mid-1990s. Studies by Rogers and colleagues (1994) indicated a high frequency of CP when the DNA demethylating agent 5-aza-2'-deoxycytidine was administered in high doses to pregnant female mice on GD 10. A similar observation was made in the rat, although there were significant differences in the developmental responses in both species (Branch et al. 1999). Bulut and colleagues (1999) noticed that congenital abnormalities, including CP, arose when 5-azacytidine (a nucleoside analog of 5-aza-2'-deoxycytidine) was administered to pregnant mice on the 11th and 14th days of pregnancy, identifying a window of susceptibility to the demethylating effects of this compound during early embryogenesis. These studies established a direct link between gene methylation and secondary palate formation. Differences in the rate of secondary palate morphogenesis have been observed in the congenic mouse pair B10/B10.A, which are genetically identical except for a 3-18 cM region on chromosome 17 that harbors the *Igf2r* imprinted locus (Melnick et al. 1998). The B10.A embryonic palates are slower growing and contain higher levels of *Igf2r* transcripts than their B10 counterparts. When exposed to equivalent amounts of corticosteroids on embryonic day 12, the B10.A strain manifested a higher frequency of CP compared with B10, and this was attributed to higher *Igf2r* expression resulting from a relaxation of methylation that permitted a switch from monoallelic to biallelic expression (Melnick et al. 1998). Overexpression of *Igf2*, an imprinted gene, in mouse embryos is frequently associated with CP (Wise and Pravatcheva 1997). Indeed, overexpression of IGF2 is observed in 10-20% of Beckwith-Wiedemann syndrome patients who present with CP in addition to several other abnormalities (Romanelli et al. 2011). Aberrant secondary palate development is occasionally observed in fragile X syndrome, in which the *FMR1* gene is transcriptionally silenced (Bastaki et al. 2004; Hagerman and Hagerman 2002). Silencing of *FMR1* occurs through a process termed methylation mosaicism, which involves both the amplification of CGG repeats and methylation of the CG dinucleotides contained therein (Giampietro et al. 1996). Kuriyama and colleagues (2008) were the first to observe changes in the methylation status of CpG islands, as well as in global DNA methylation, in the secondary palates of mice born to mothers exposed to all-trans retinoic acid at doses that induced CP. Using restriction landmark genome sequencing, they identified six genes that were differentially expressed, presumably due to differential gene methylation, between control and all-trans retinoic acid–treated mice. Studies on A/J mice treated with teratogens such as dilantin, corticosteroid, and 6-aminonicotinamaide have identified the *Nat2* gene (expressing *N*-acetyltransferase 2) as a possible culprit in such teratogen-induced orofacial clefting (Erickson 2010). The substrate for Nat2, *p*-aminobenzoylglutamate, is a breakdown product of folate metabolism (which generates methyl groups for various biochemical reactions), thereby linking DNA methylation to clefting induced by these teratogens. Except for the aforementioned reports, the epigenetic components underlying secondary palate development and their contribution to CP have only recently begun to attract significant research attention. Specifically, no information exists about the methylome of the embryonic secondary palate. Identifying genes whose promoters undergo differential methylation during development of the palate will be a significant step toward identification of genes that are uniquely susceptible to environmental perturbation. Furthermore, little is known regarding the role miRNAs play during development of the palate. We have, therefore, recently begun to define global promoter methylation profiles during development of the murine secondary palate as a prelude to identifying differentially methylated genes and expressed miRNAs in this tissue.

Role of DNA Methylation in Murine Secondary Palate Development

Basic Features and Current Concepts

DNA methylation involves the transfer of a methyl group from S-adenosyl methionine to carbon-5 of a cytosine ring resulting in 5-methylcytosine, often referred to as the fifth base. Cytosine methylation typically occurs in CpG dinucleotide sequences (Jones and Takai 2001) but also occurs in non-CpG residues (CpA, CpC, and CpT) (Barrès et al. 2009; Laurent et al. 2010). More recently, 5-hydroxy methylcytosine (Kriaucionis and Heintz 2009; Tahiliani et al. 2009), a product formed by the oxidation of 5- methylcytosine by ten eleven translocation proteins, has been garnering considerable attention because of its critical role in DNA methylation

reprogramming in the mammalian zygote (Iqbal et al. 2011; Wossidlo et al. 2011). Analysis of embryonic stem cells indicates strong enrichment of 5-hydroxy methylcytosine at transcriptional start sites associated with bivalent chromatin of developmentally regulated genes (Pastor et al. 2011).

CpG methylation, however, remains the best-studied and predominant type of DNA methylation (Bird 1980). CpG residues tend to concentrate in genomic regions known as CpG islands, which have a G/C content of 55% or greater and are most often located in the promoter regions of many genes. Methylation of CpG islands in gene promoters correlates with transcriptional silencing (Antequera 2003; Caiafa and Zampieri 2005) by means of either inhibition of transcription factor binding (Geiman and Robertson 2002) or interaction of methyl-CpG binding proteins associated with transcriptional repression (Deaton and Bird 2011; Meehan et al. 1992). Promoter-associated CpG islands of housekeeping genes tend to remain unmethylated, thus facilitating active gene transcription. On the other hand, for tissue-specific genes, these islands can exhibit differential methylation such that the genes are variably expressed in different tissues. Nearly 50% of tissue-specific genes possess CpG islands (Suzuki et al. 2001), and CpG island methylation patterns for each tissue or cell type are unique (Shiota et al. 2002). Indeed, differential methylation of promoter-associated CpG islands has long been thought to be the mechanism of tissue-specific gene regulation. Recent studies, however, that demonstrate that regions outside CpG islands, termed CpG island shores, harbor differentially methylated regions (DMRs[1]) that dictate tissue specificity have prompted reexamination of this paradigm (Doi et al. 2009; Irizarry et al. 2009). It is also becoming increasingly apparent that CpG residues critically involved in gene regulation have a propensity to be located in CpG-sparse regions (Eckhardt et al. 2006; Nagae et al. 2011; Schmidl et al. 2009; Sun et al. 2011; Yuen et al. 2011).

Tissue-specific DNA methylation patterns are precisely programmed during embryogenesis (Kafri et al. 1992; Monk et al. 1987). Failure to establish correct methylation patterns can lead to embryonic lethality (Li et al. 1992) or can result in developmental craniofacial malformations (Abu-Amero et al. 2008; Gonzales et al. 2005; Kakutani et al. 1996; Matsuda and Yasutomi 1992; Ohgane et al. 2001) including CP (Bliek et al. 2008; Kuriyama et al. 2008; Loenarz et al. 2010). Previous studies in our laboratory, utilizing messenger RNA (mRNA[1])–based microarray analysis, have demonstrated the expression of a number of methyltransferases and methyl-CpG binding proteins in GD 9.5 murine embryonic craniofacial tissue (Mukhopadhyay et al. 2006a). This provides evidence that DNA methylation is a dynamic process occurring during secondary palate development.

The Murine Secondary Palate Methylome

To characterize the methylome in the developing murine secondary palate, we determined global promoter methylation profiles in the secondary palate on GDs 12, 13, and 14 (representing the gestational period during which the palate forms) (Figure 1). A two-step experimental strategy was employed: first, genomic DNA fragments, enriched for methylated CpG residues, were isolated from the secondary palate and converted into hybridization probes; second, these probes were hybridized to murine promoter microarrays (Seelan et al., unpublished data) (Figure 2). Regions containing at least six contiguously methylated probes within a 900–base pair

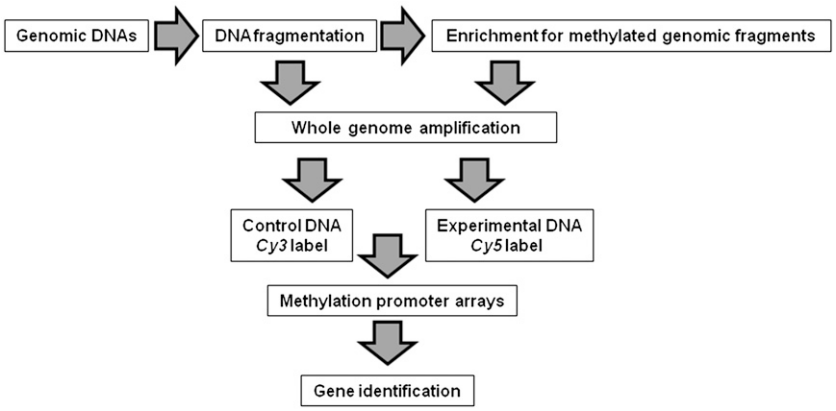

Figure 2 A schematic outline to identify differentially methylated genes using promoter microarrays. Genomic DNAs from secondary palates (gestation days 12–14) were isolated and fragmented by sonication. Sonicated DNA was incubated with MBD2b, a methyl-CpG binding protein (Active Motif, Inc., Carlsbad, CA), for enrichment of methylated genomic fragments. The enriched DNA fragments from all three gestational days were amplified by whole genome amplification. Genomic DNAs not subjected to methylation enrichment were used as controls. Control DNA, which was labeled with Cy3, and experimental DNA, which was labeled with Cy5, were hybridized to NimbleGen 2.1 M mouse promoter microarrays (Roche NimbleGen, Madison, WI). Nine microarrays representing three biologic replicates on each of gestation days 12, 13, and 14 were probed. After hybridization and washing, the microarray chips were scanned and the accrued data were analyzed using biostatistical methods (Seelan et al., unpublished data).

Table 1 Characteristic features of the secondary palate methylome during development

	GD 12	GD 13	GD 14
Number of MRIs	4423	6195	6102
Number of methylated genes[a]	4078	5328	5252
Number of uniquely methylated genes	17	405	344
Mean length of MRIs, %[b]	89	100	96
% CpG islands[c]	9.5	11.6	10.5

GD, gestational day; MRI, methylated regions of interest.
[a]Genes associated with MRIs.
[b]Expressed as a percentage of the mean MRI length on GD 13.
[c]Overlapped by at least one MRI.

region were identified and designated as methylated regions of interest. Based on this criterion, approximately 5600 genes were found to be methylated, with a majority showing little evidence of differential methylation during palate development (GDs 12–14) (Table 1). Methylated regions of interest were observed to occur predominantly in intragenic regions (i.e., gene bodies that include 3' untranslated regions, 5' untranslated regions, exons, and introns) rather than in promoters and upstream regions of genes. This finding is consistent with studies of the human methylome, wherein methylated regions of interest were found predominantly in gene bodies (approximately 50%), whereas only approximately 6% occurred at the 5' end of genes (Rauch et al. 2009). In contrast with genes methylated in promoters, which are often associated with decreased gene expression, intragenic methylation tends to be positively correlated with transcription (Rauch et al. 2009). Although the significance of intragenic methylation is yet to be elucidated, it has been suggested that it may repress expression of antisense transcripts, inhibit transcription from alternate mRNA start sites, modify transcription efficiency, and/or bring about conformational changes in histones (Ball et al. 2009; Shenker and Flanagan 2012; Suzuki and Bird 2008).

When gene ontology analysis using the Database for Annotation, Visualization and Integrated Discovery was performed to identify signaling pathways affected by genes methylated during palate development (Seelan et al., unpublished data), several categories associated with palatogenesis were readily apparent. These include apoptosis, cell adhesion, signaling systems mediated by Wnt, Hedgehog, and Notch, and the biosynthesis of proteoglycans necessary for ECM formation (see "Introduction"). Thus, DNA methylation appears to be critical for several processes that underlie palate morphogenesis. The secondary palate methylome that we have broadly characterized is currently being evaluated by pyrosequencing techniques to determine methylation levels of individual CpG residues localized within the methylated regions of interest of specific genes. These analyses are expected to provide robust evidence of differential methylation, if any, in genes during palate development from GDs 12 to 14. The secondary palate methylome should be a valuable reference for examining the role environment plays in the etiology of CP.

The *Sox4* Paradigm

Sox4 is widely expressed in the murine embryo (Dy et al. 2008) and regulates multiple aspects of neural crest cell development (Hong and Saint-Jeannet 2005). Although its role in palatal development or CP is not known, *Sox4* has been implicated as a candidate gene for human CL with CP (Juriloff and Harris 2008). Sox4 is a transcription factor belonging to the SoxC family and is functionally associated with the TGFß and Wnt-ß-catenin signaling pathways (Scharer et al. 2009). Significant downregulation of *Sox4* gene expression occurs in developing palatal tissue between GD 12 and GD 13 and remains steady thereafter from GD 13 to GD 14 (Mukhopadhyay et al. 2006a). This expression pattern could be due to critical CpG residues in the upstream regulatory region of *Sox4* becoming increasingly methylated from GD 12 to GD 13.

To test this premise, the CpG methylation profile of the upstream region of *Sox4* was determined by bisulfite sequencing of genomic DNAs obtained from GDs 12, 13, and 14 secondary palatal tissue (Greene and Pisano 2010). Six polymerase chain reaction amplicons from bisulfite-modified DNA were generated corresponding to approximately 2 kb of the *Sox4* upstream region (relative to the ATG start codon) (Figure 3) and were sequenced to determine the methylation levels of nearly all CpG residues. Comparison of percentage methylation levels of these amplicons from the three gestational days revealed significant methylation differences in amplicons 3 and 4 only from GD 12 to GD 13, but not between GD 13 and GD 14 (Figure 3). None of the four remaining amplicons displayed any significant change in methylation levels on any GD, and methylation levels of all amplicons on GD 13 were almost identical to their counterparts on GD 14 (Figure 3**).** Interestingly, the two amplicons that exhibited differential methylation between GD 12 and GD 13 were located in regions outside the CpG island of the *Sox4* gene (which spans amplicon 1), consistent with their presence in CpG island shores (Irizarry et al. 2009). The two amplicons, therefore, represent putative *Sox4* DMRs. Notably, the CpG island of the *Sox4* promoter remained unmethylated on all three gestational days, with methylation levels ranging 0–1.5% (Figure 3). The distal

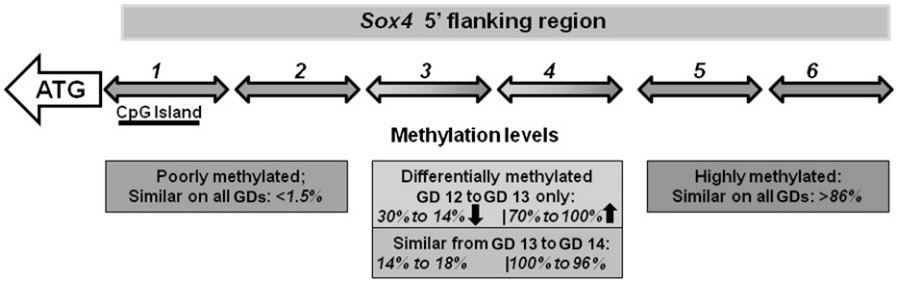

Figure 3 CpG methylation profiling of murine *Sox4* upstream region. Six polymerase chain reaction amplicons (numbered 1-6) representing the *Sox4* upstream region (relative to the ATG start site) were generated from genomic DNAs isolated from gestational days (GDs) 12 to 14 secondary palate for CpG methylation profiling. CpG profiling was undertaken by amplifying bisulfite-modified DNA (Clark et al. 1994) and sequencing the cloned amplicons. Methylation levels of each CpG residue were inferred by sequencing approximately 10 clones. Amplicons 1 and 2 are poorly methylated (0-1.5%) and amplicons 5 and 6 are heavily methylated (86-96%) on all three GDs. Amplicons 3 and 4 are differentially methylated from GD 12 to GD 13 (upper box) but not from GD 13 to GD 14 (lower box). Note that methylation decreases from GD 12 to GD 13 in amplicon 3 and increases from GD 13 to GD 14 in amplicon 4. Changes in methylation levels in amplicon 4 are consistent with *Sox4* messenger RNA expression (see text). No significant changes were observed in methylation levels in amplicons 3 and 4 from GD 13 to GD 14.

DMR (amplicon 4) contained CpG residues that showed significant increase (>30% change) in methylation levels from GD 12 to GD 13 (data not shown), which correlated with a decrease in *Sox4* mRNA levels during these developmental time points; no significant change in methylation levels was observed in these CpG residues between GD 13 and GD 14. On the other hand, the proximal DMR (amplicon 3) contained CpG residues that showed a significant decrease in methylation from GD 12 to GD 13, which was inconsistent with decreasing *Sox4* mRNA levels detected in the secondary palate (Mukhopadhyay et al. 2006a). Efforts are now underway to assess the functional relevance of these CpG residues in *Sox4* expression in the palate. Thus, detailed CpG profiling of the upstream sequence of *Sox4*, a candidate gene likely involved in murine palatogenesis, reveals that the period of dramatic change in DNA methylation occurs between GD 12 and GD 13, that CpG islands are predominantly unmethylated and exhibit no differential methylation during palatogenesis, and that DMRs likely associated with palate development are localized in CpG-sparse shore regions. *Sox4* could be one of the few genes that exhibit differential methylation during secondary palate development.

MicroRNAs

MicroRNAs and Embryogenesis

MicroRNAs are a unique class of gene silencers discovered nearly two decades ago that belong to the largest family of small, noncoding RNAs in plant and animal systems (Lee et al. 1993; reviewed in Wang et al. 2007; Wightman et al. 1993; Zhang et al. 2007). MicroRNAs, existing either in polycistronic clusters or within the introns of protein-coding genes, are transcribed as long primary miRNAs (Bartel 2009; Chuang and Jones 2007). Processing of miRNAs into double-stranded, stem-loop precursor miRNAs occurs in the nucleus by means of a protein complex comprised of a type 3 ribonuclease (Drosha) (Lee et al. 2004). Following transport to the cytoplasm, these precursor miRNAs are processed further by Dicer, another type 3 ribonuclease, into mature, functional miRNAs (Lund et al. 2004). The mature miRNAs are subsequently incorporated in RNA-induced silencing complexes (Lee et al. 2002), which can silence numerous, downstream gene targets by binding to specific sequences in the 3' untranslated regions or within the coding regions (Tay et al. 2008). Although such binding usually silences expression of target genes by means of either inhibition of translation or mRNA destabilization (Bartel 2009; Pan and Chegini 2008), recent studies also document miRNAs as translation stimulators (Vasudevan et al. 2007). Experimental findings reveal that complete complementarity of binding between miRNAs and gene targets, although rare in animals, can result in degradation of the target (Meister 2007), whereas partial complementarity triggers translational repression (Kim and Nam 2006). Current estimation indicates that miRNAs account for approximately 1% of predicted genes in higher eukaryotic genomes and that approximately 30% of protein coding genes are regulated by miRNAs (Ross et al. 2007).

Over the past several years, numerous studies have established miRNAs as critical regulators of proliferation, differentiation, and apoptosis—cellular events indispensable for proper embryonic development (reviewed in Conrad et al. 2006; Lee et al. 2006; Mineno et al. 2006). During organogenesis, expression of avian (Darnell et al. 2006), zebrafish (Giraldez et al. 2005), and murine (Takada et al. 2006) miRNAs are tightly regulated in a spatiotemporal manner (Saetrom et al. 2007; Wienholds et al. 2005). Loss of the miRNA-processing enzyme Dicer1 in mice leads to embryolethality (Bernstein et al. 2003; Yang et al. 2005). Inactivation of Dicer in *Caenorhabditis elegans* and in zebrafish results in heterochronic phenotypes (Grishok et al. 2001) and overall growth arrest during embryogenesis (Wienholds et al. 2003), respectively. The indispensability of

miRNAs to stem cell proliferation and differentiation during embryogenesis has been further demonstrated by the failure of differentiation of embryoid body-like structures formed by Dicer1-deficient embryonic stem cells and by the rescue of the differentiation defect in Dicer1-reconstructed clones (Bernstein et al. 2003; Kanellopoulou et al. 2005). The importance of the maintenance and regulation of endogenous miRNA levels during mammalian neurogenesis and neurodevelopment has also been reported (Giraldez et al. 2005; Hosako et al. 2009). Monoallelic deletion of murine *dgcr8*, a gene encoding an RNA-binding protein required for miRNA biogenesis, resulted in abnormal postnatal development of prefrontal cortical circuitry, which eventually led to cognitive deficits (Schofield et al. 2011). Cardiomyocyte-specific deletion of *dgcr8* revealed a fully penetrant cardiomyopathy phenotype and premature lethality, which highlights the vital roles of miRNAs in maintaining cardiac function (Rao et al. 2009). These studies reinforce the centrality of miRNAs in orchestrating normal embryogenesis.

MicroRNA Gene Expression Signature of the Developing Orofacial Tissue

The physiologic roles of miRNAs in orofacial (including secondary palate) development have only recently begun to be examined. In zebrafish, miRNA 140 has been reported to inhibit PDGF receptor α–mediated attraction of cranial neural crest cells to the oral ectoderm (Eberhart et al. 2008), a process necessary for normal morphogenesis of the secondary palate. Lately, an intriguing hint that polymorphisms in human miRNA-coding genes might underlie the etiology of CP was presented by the association of an SNP in the human gene encoding miR-140 with isolated CP in a small cohort from western China (Li et al. 2010). An array of morphogens, growth factors, and signaling mediators, such as members of the TGFß family, bone morphogenetic proteins (BMPs[1]), Wnts, Shh, epidermal growth factor, mitogen-activated protein kinases, cyclic adenosine monophosphate (cAMP), and retinoic acid, as well as a variety of downstream transcription factors, such as Sma and MAD related family proteins (Smads), cAMP response element-binding protein (CREB), CREB binding protein, E1A binding protein p300, Sloan-Kettering Institute protein (Ski), Ski-related novel protein N, nuclear receptor corepressors, and histone deacetylases, have been reported to interact and coordinate the dynamic process of orofacial ontogenesis (Brunet et al. 1995; Gehris and Greene 1992; Lan et al. 2006; Mendelsohn et al. 1994; Mukhopadhyay et al. 2006a, 2006b; Warner et al. 2002; Weston et al. 1998). Many of the aforementioned signaling mediators and transcriptional regulators have been shown to be targeted by an ever-growing number of miRNAs (Kawasaki and Taira 2003; Kennell et al. 2008; Li and Carthew 2005; Lin et al. 2009; Martello et al. 2007; Zhao et al. 2008). For instance, chondrocyte-specific miRNA 140 was shown to regulate endochondral bone development by BMP signaling, and loss of miR-140 expression resulted in orofacial deformities (Nakamura et al. 2011). In another study, TGFß-mediated EMT, a process critical to normal development of the secondary palate (Sun et al. 1998), was shown to be associated with marked downregulation of miR-205 and all five members of the miRNA 200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) (Gregory et al. 2008). Remarkably, induced expression of the miR-200 family alone was sufficient to prevent TGFß-induced EMT.

Interestingly, miRNAs can also function as effectors of key signaling mediators (such as, TGFßs, BMPs, and Wnts, among others), governing diverse cellular events essential for orofacial development. For example, miR-335, regulated by Wnt signaling, orchestrates mesenchymal stem cell proliferation, migration, and differentiation (Tomé et al. 2011). Regulation of cellular differentiation by BMP signaling, by modulating the expression of members of the miRNA-17-92 cluster, has also been demonstrated (Wang et al. 2010). Furthermore, recent studies reported TGFß-induced myofibroblast differentiation and collagen expression by means of miR-21 and miR-29, respectively (Maurer et al. 2010; Yao et al. 2011).

A recent study defined unique signatures of hundreds of miRNAs expressed within developing orofacial tissue and identified several developmentally regulated clusters of miRNAs that target genes that encode proteins associated with cellular proliferation, adhesion, differentiation, apoptosis, and EMT, all processes central to normal orofacial morphogenesis (Mukhopadhyay et al. 2010). Findings from this study revealed that out of 588 murine miRNA genes examined, 68, 72, and 66 miRNAs were found to be expressed in the developing orofacial region on GD 12, GD 13, and GD 14, respectively. Among these groups, 57 were common to all three days of gestation. A number of differentially expressed miRNAs (such as miR-20a, miR-20b, miR-193, miR-193b, miR-140, members of the let-7 family of miRNAs, miR-152, miR-122, and miR-206, among others) were identified by target analyses as potentially vital modulators of downstream gene targets that encode proteins that execute pivotal functions in orofacial development. Target analyses with multiple miRNA target prediction software programs (e.g., miRDB: http://mirdb.org/miRDB/; PicTar: http://pictar.mdc-berlin.de; TargetScan: www.targetscan.org; and, miRanda: www.microrna.org/microrna/home.do), demonstrated that within the growing orofacial tissue various developmentally regulated miRNAs could potentially target an array of cytoskeletal proteins, growth and differentiation factors, signal transduction modulators and effectors, and transcription factors, all documented to play crucial roles in orofacial ontogeny (Figure 4).

To investigate cause–consequence relationships between developmentally regulated miRNAs and their putative gene targets, ingenuity pathway analysis of miRNA gene expression profiling data was performed to assign these miRNAs to specific cellular pathways (Mukhopadhyay et al. 2010). One representative gene network is noteworthy for connecting eight differentially expressed miRNAs (miR-20a, miR-20b, miR-22, miR-206, miR-362, miR-106a, miR-152, and miR-140) and associating these with important physiologic events, such as cellular proliferation, growth of epithelial

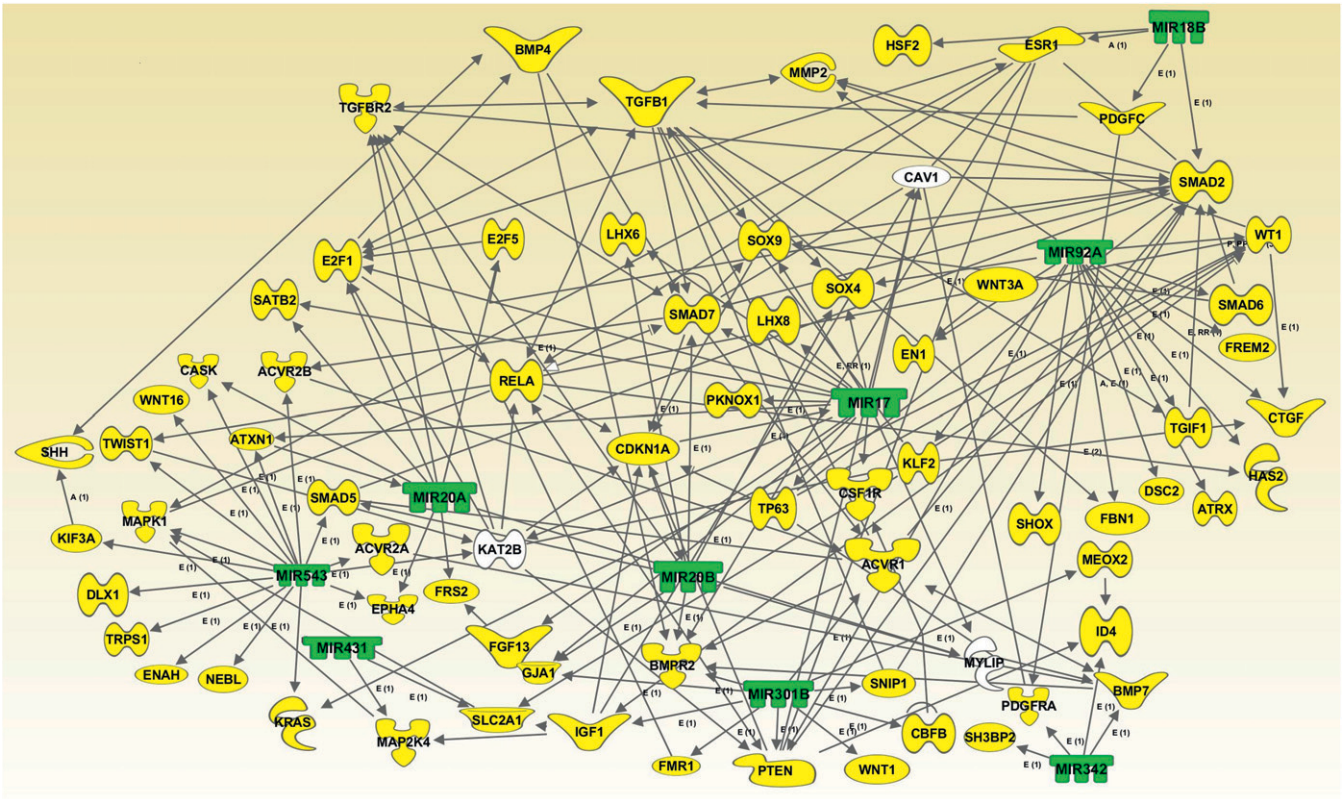

Figure 4 Computational gene interaction predictions: gene network with microRNAs demonstrating diminished expression in developing orofacial tissue on gestational day (GD) 13 versus GD 12. A network with selected genes encoding microRNAs (green) with decreased expression on GD 13 versus GD 12 developing orofacial tisue and their known or predicted target genes (yellow) critical for orofacial ontogenesis was constructed with ingenuity pathway analysis software and the miRDB (http://mirdb.org/miRDB/) database. Solid lines specify direct relationships, whereas dotted lines indicate indirect interactions (Mukhopadhyay et al. 2010).

cells, and cell survival (Figure 5). The importance of this network is further emphasized by the presence of several direct and indirect gene targets (of the differentially expressed miRNAs) reported to be expressed and to execute (or likely to execute) crucial roles during orofacial ontogenesis. Examples of such target genes include *CREBBP* (Warner et al. 2004), *CBP/p300* (Warner et al. 2006), *Cdkn1a/p21* (Yu et al. 2008), and *Bcl2*, *Cux1*, *Foxc1*, and *Mybl1* (Mukhopadhyay et al. 2006a, 2006b). Furthermore, a range of signal transduction pathways, most of which are engaged in normal orofacial development, can be linked directly or indirectly to various miRNAs and genes present in the network (not shown). Examples of such relevant pathways include the TGFß-, BMP-, Wnt-, retinoic acid-, janus tyrosine kinase/signal transducer and activator of transcription, vascular endothelial growth factor, phosphoinositide 3-kinase/protein kinase B, and calcium-signal transduction pathways (not shown). Such gene interaction maps firmly underscore the paramount importance of a range of developmentally regulated miRNAs in orchestrating orofacial ontogenesis by modulating expression of crucial, downstream gene targets.

Finally, the importance of the miRNAs differentially expressed within the embryonic orofacial region in governing expression of key genes indispensable for orofacial morphogenesis is convincingly highlighted by two miRNA–mRNA gene networks developed with ingenuity pathway analysis and the miRDB online database (Mukhopadhyay et al. 2010). It is noteworthy that these networks that display either diminished (Figure 4) or enhanced (not shown) expression within the developing orofacial tissue directly link potential gene targets reported to be crucial for development of the orofacial region. Examples of some of these gene targets are TGFßs and BMPs (Brunet et al. 1995; Zhang et al. 2002), *Meox-2* (Jin and Ding 2006), *PDGF* (Choi et al. 2009), matrix metalloproteinases (Blavier et al. 2001; de Oliveira Demarchi et al. 2010), *Cdkn1a/p21* (Yu et al. 2008), *Satb2* (Leoyklang et al. 2007), and *Cask* (Laverty and Wilson 1998). Collectively, these studies emphasize that differentially expressed miRNAs, which regulate crosstalk among various signaling cascades, orchestrate differentiation and morphogenesis of developing orofacial tissue such as the secondary palate. Because individual miRNAs may target multiple downstream genes, complex, cooperatively interacting regulatory combinations are likely to exist.

Concluding Remarks

Epigenetic studies that pertain to secondary palate development are still in a nascent stage and represent a fertile area

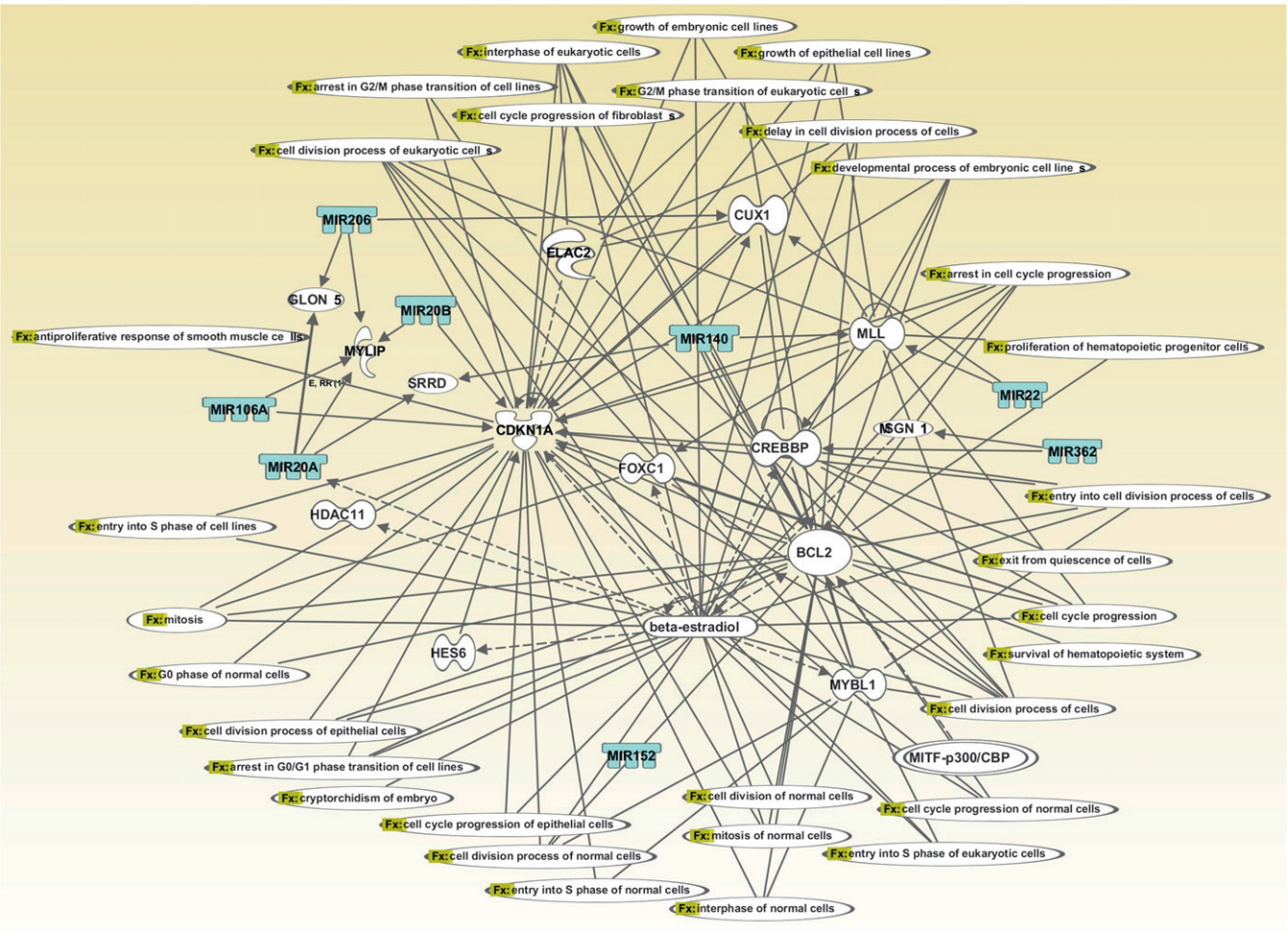

Figure 5 Computational gene interaction predictions: selected microRNA gene network in developing orofacial tissue. A network with selected genes encoding microRNAs was constructed with ingenuity pathway analysis software. Several differentially regulated microRNA genes from the study were used to construct a gene association map (Figure 4) for predicting various cellular and molecular events operative within the developing mouse orofacial tissue. Solid lines specify direct relationships, whereas dotted lines indicate indirect interactions (Mukhopadhyay et al. 2010).

for intensive investigation. Identification and functional validation of genes whose expression is driven by differential methylation of promoter regions and determination of the functional role of miRNAs during palatogenesis will provide deep insights into the mechanistic basis by which environmental influences impact secondary palate development. Studies that examine chromatin conformation states at the genome-wide level during secondary palate development are currently lacking. Such studies have the potential to identify "poised" developmental genes (Pastor et al. 2011) that are primed for expression during secondary palate development and therefore likely to play crucial roles in the etiology of CP. A pertinent observation here is that neural tube defects and orofacial clefts, including CP, are often seen when pregnant mothers are exposed to valproic acid (Ornoy 2009), an antiepileptic drug and a known human teratogen. The teratogenicity of this drug is likely due to its ability to inhibit histone deacetylases, whose function is to reduce the acetylation of histones, thereby altering chromatin conformation states that affect gene transcription (Phiel et al. 2001). The implication of this finding is that inappropriate chromatin conformation induced in the early embryo/fetus can impair craniofacial development, including secondary palate/orofacial tissue development. A comparatively larger body of literature exists for CL/P, which occurs much more frequently than CP, but extensive epigenetic studies are likewise lacking for understanding the etiology of this disorder. However, a growing body of evidence clearly suggests that epigenetic processes contribute to the etiology of this order (Bliek et al. 2008; Plamondon et al. 2011; Spritz 2001).

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