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The role of epigenetics and miRNAs in orofacial clefts

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Abstract

Orofacial clefts (OFCs) have multiple etiologies and likely result from an interplay between genetic and environmental factors. Within the last decade, studies have implicated specific epigenetic modifications and non-coding RNAs as additional facets of OFC etiology. Altered gene expression through DNA methylation and histone modification offer novel insights into how specific genes contribute to distinct OFC subtypes. Epigenetics research has also provided further evidence that cleft lip only (CLO) is a cleft subtype with distinct etiology. Polymorphisms or misexpression of genes encoding microRNAs, as well as their targets, contribute to OFC risk. The ability to experimentally manipulate epigenetic changes and non-coding RNAs in animal models, such as zebrafish, *Xenopus*, mice, and rats, has offered novel insights into the mechanisms of various OFC subtypes. Although much remains to be understood, recent advancements in our understanding of OFC etiology may advise future strategies of research and preventive care.

1. Introduction

Human craniofacial development is an intricate process that occurs during the first ten weeks of gestation (Wilderman, VanOudenhove, Kron, Noonan, & Cotney, 2018). It involves a tightly orchestrated series of developmental events including morphogenesis, cellular differentiation, and molecular signaling. Misregulation of any of these processes may result in orofacial clefts (OFCs) which effect 1 in 700 births on a global average (Mossey & Catilla, 2003). OFCs, which refer to any cleft of the mouth, face, and/or nose, are among the most common birth defects. Non-syndromic OFCs, which represent approximately 70% of all OFC cases, refers to those in which the cleft presents in the absence of other associated congenital effects. This category has been traditionally divided into two main groups that

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reflect our best understanding of OFC etiologies: cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). More recently, epigenetics studies have indicated that cleft lip only (CLO) may have its own etiology among non-syndromic OFCs (Sharp et al., 2017). Although OFCs can be corrected, this condition brings with it a considerable financial, psychological, and social cost (Wehby & Cassell, 2010). The best approach would be to prevent OFCs whenever possible, thus making OFC etiology a high priority in developmental biology and birth defect research.

Some of the known risk factors for OFCs include polymorphisms of genes that influence orofacial development, parental lifestyle and related health conditions (i.e., smoking, obesity, and diabetes) (Åberg, Westbom, & Källén, 2001; Blanco, Colombo, & Suazo, 2015; Health & Services, 2014), pharmaceuticals and nutritional supplements (G. Shaw, 1995; G. M. Shaw, Wasserman, O'Malley, Tolarova, & Lammer, 1995), and environmental exposure to chemicals (Spinder et al., 2017). It is suspected that many OFC cases result from a combination of these factors. While it is known that complex gene-environment (G x E) interactions influence orofacial development, relatively little is understood about the mechanisms that direct the outcome of this process (Dixon, Marazita, Beaty, & Murray, 2011). Studies attempting to identify G x E interactions that cause OFCs have historically reached divergent conclusions (Chung, Kowalski, Kim, & Buchman, 2000; Health & Services, 2014; Wehby & Murray, 2010; Wyszynski, Duffy, & Beaty, 1997).

More recent work toward understanding the regulation of gene expression through epigenetic modifications and non-coding RNAs has offered an entirely new perspective of OFC etiology with greater resolution. In particular, these may explain previously observed inconsistencies and divergent findings (Schoen et al., 2017; Sharp et al., 2017). Furthermore, some of these mechanisms are known to be influenced by environmental factors (Seelan, Pisano, & Greene, 2019) (a companion review by Garland et al. in this issue). In this review, we summarize the current standing of the field regarding epigenetic and microRNA-related mechanisms of OFCs. We focus primarily on studies that elucidate functional mechanisms of these processes including cell and organ culture manipulations, animal studies, and large scale genome- or epigenome-wide studies in rodents and humans, although we consider other studies that offer additional insight toward these mechanisms. We searched PubMed and Web of Science databases using the following concepts: orofacial, cleft lip, cleft palate, epigen*, methylation, acetylation, histone, and miRNA. We included studies that specifically relate to mechanisms of OFCs, with a focus on animal or cell culture experiments and human genome-wide association studies.

2. Epigenetic mechanisms of orofacial clefts

Gene expression can be altered by modifications to the genome that do not involve changes in DNA nucleotide sequence. Known as epigenetic modifications, these add an additional layer of information to the genetic code that can be transgenerationally conserved (Kiefer, 2007). The best understood epigenetic modifications in animals include DNA methylation, which can alter gene expression in mammals, and histone modifications, which regulate the accessibility of chromatin to transcriptional machinery. Cells are epigenetically programmed toward specific identities and fates through lineage-specific gene silencing, giving rise to

diverse tissues throughout the body (Cantone & Fisher, 2013; Godini, Lafta, & Fallahi, 2018). Consequently, the epigenetic landscape of a given cellular genome is dynamic and has natural variability that occurs during developmental processes (*e.g.*, craniofacial and neural tube development) (N. D. Greene, Stanier, & Moore, 2011; Varrela, 1991), tissue regeneration (Katsuyama & Paro, 2011), and senescence (Horvath, 2013). Genomic imprinting, in which genes are transgenerationally expressed on a parent-of-origin basis, involves both DNA methylation and histone modifications (Ferguson-Smith, 2011). Malignancies and health conditions, such as cancers and diseases associated with aberrant imprinting, also affect or may be caused by epigenetic dynamics (Portela & Esteller, 2010). At the organismal and cellular levels, environmental factors influence epigenetic modifications in ways that can lead to altered developmental outcomes (Feil & Fraga, 2012). Many cases of non-syndromic OFCs, including CL/P and CPO, are suspected to be the result of gene-environment interactions (Dixon et al., 2011; Jambhekar, Dhall, & Shi, 2019; Seelan et al., 2019). Epigenetic modification could therefore explain, at least partly, variability of OFC prevalence between human populations that are otherwise not explained by genotypic differences.

In this section, we will discuss the field's current understanding of how epigenetic modifications influence non-syndromic OFCs in humans and animal models. We selected articles for this review based on their relevance to OFCs and epigenetics as well as their role in advancing mechanistic understanding of the topic. Environmental factors, particularly folates and retinoids, are considered where relevant toward understanding the role of epigenetics in OFCs. Due to space constraints, we refer readers to another review for the role of genomic imprinting on OFCs (Seelan et al., 2019). Other environmental mechanisms contributing to OFCs are examined in greater detail in a companion review (Garland et al., in this issue).

2.1. Nucleic acid methylation

As described above, DNA methylation pervasively underlies the regulation of cellular processes relating to animal development and physiology. In mammals and other vertebrates, it is generally accepted that methylation of cytosine (C) on the C5 position, resulting in 5-methylcytosine (5mC), is the only epigenetic form of DNA methylation. To date, the DNA methylation of adenine (A) is controversial in vertebrates (discussed at the end of this section), while methylation of guanine (G) and thymine (T) are undescribed. C-methylation (methylation from here on) is catalyzed by DNA methyltransferases (DNMTs) which rely solely on the pool of S-adenosylmethionine (SAM) to supply methyl groups (Lei et al., 1996). Consequently, the methyltransferase activities of DNMTs (as well as histone-modifying enzymes) are dependent on adequate SAM generated by the folate and methionine cycles (Seelan et al., 2019).

2.1.1. Mechanisms of DNA methylation—Methylation is thought to facilitate gene silencing by preventing transcriptional machinery or activators from binding to these regions through spatial hindrance. Additionally, recruitment of methyl-CpG binding proteins to 5mC may interact with and engage the activities of transcriptional repressors such as histone deacetylases (Du, Luu, Stirzaker, & Clark, 2015). Methylation dynamics generally occurs

within the *cis*-regulatory elements, especially in the promoter and enhancer regions, to control variation in gene expression (Jones, 2012). The promoter is proximally adjacent to the 5'-untranslated region (5'-UTR) of the gene and is where transcriptional machinery binds to DNA to initiate transcription. Enhancers may be proximal to the promoter or distal to the 3'-untranslated region (3'-UTR) and are bound by additional transcription factors to promote gene expression. The enhancer may be physically looped to be positioned near, and interact with, the promoter to facilitate transcriptional activation (Whalen, Truty, & Pollard, 2016; Williamson, Hill, & Bickmore, 2011). Enhancer activity is strongly tissue-dependent and is particularly important for spatiotemporal dynamics of gene expression during embryogenesis (Ong & Corces, 2012). Methylation dynamics within gene bodies are also correlated with gene expression (Blattler et al., 2014). For reasons described in the next paragraph, many DNA methylation studies focused primarily on the promoter regions (Jones, 2012), although there is increasing evidence that enhancer and gene body methylation may have similar or possibly greater importance for regulating developmental gene expression.

Methylation motifs: Double-stranded DNA methylation occurs at specific base sequences (motifs) to transgenerationally regulate the expression of specific genes (Figure 1). Epigenetic methylation is often observed in genomic regions enriched with 5'-CpG-3' motifs called CpG islands (Jones, 2012). Methylation at CpG islands is symmetrically maintained on both strands making it a heritable epigenetic mark that does not rely on *de novo* methylation for re-establishment. Promoters are strongly associated with CpG islands; in contrast, enhancers may or may not be associated with CpG islands. Because of the promoter-CpG island correlation, earlier studies focused primarily on promoter methylation as the major epigenetic control over the regulation of gene expression. Another epigenetic marker can be found within the pentanucleotide motif, 5'-CCWGG-3' (where W may be either A or T) (Figure 1), which undergoes methylation at the internal C (Lorincz & Groudine, 2001). Methylation of 5'-CWG-3' was transgenerationally conserved in mammalian cells *in vivo* (Clark, Harrison, & Frommer, 1995), and it is thought that this was due to its recognition as 5'-CCWGG-3' by methyltransferase (Lorincz & Groudine, 2001). The stable methylation of this motif challenged prevailing theory that CpG islands contained the only C bases that could be transgenerationally marked. 5'-CCWGG-3' appears to be particularly important for enhancer methylation dynamics in orofacial development (X. Shu, S. Shu, H. Cheng, et al., 2018; Shu, Shu, Zhai, Zhu, & Ouyang, 2018), a topic that is now gaining increasing attention.

Adenine methylation: While 5mC is usually regarded as the only DNA methylation in mammals, this paradigm has been challenged with evidence supporting the existence of methylated A as an epigenetic marker (Wu et al., 2016). A-methylation, which converts A nucleobases into N⁶-methyldeoxyadenine (N⁶mA), is a confirmed nucleobase modification in mammalian RNA processing (as the RNA counterpart N-6-methyladenosine, or m⁶A) (Geula et al., 2015) but its role in mammalian or vertebrate DNA is controversial. A-methylation of DNA is observed in prokaryotes and some eukaryotes, including protists and *Caenorhabditis elegans*, where it is part of a transgenerational control mechanism over mitochondrial stress adaptation in the latter species (C. Ma et al., 2019). More recent studies

have refuted claims of N⁶mA playing an epigenetic or functional role in mammalian DNA methylation; one study suggests sample contamination by RNA or bacteria along with technical limitations (Douvlataniotis, Bensberg, Lentini, Gylemo, & Nestor, 2020), whereas another provides evidence that N⁶mA is misincorporated into the genome by DNA polymerase following the processing of ribo- N⁶mA by the nucleotide salvage pathway (Musheev, Baumgartner, Krebs, & Niehrs, 2020). Given these circumstances, A-methylation in DNA is a difficult epigenetic mechanism to prove.

In a review of nucleic acid methylation and orofacial development, Seelan et al. (2019) briefly discuss the possible role for A-methylation in RNA (Seelan et al., 2019). RNA modifications typically involve N⁶mA methylations mediated by six members of the *Nsun* family of genes (*Nsun2* through *Nsun7*) that contain the NOL1/NOP2/sun domain (Chi & Delgado-Olguin, 2013; Yue, Liu, & He, 2015). Most evidence for the involvement of *Nsun* genes in orofacial development is derived from the high expression of their family members in the first branchial arch (BA1) of mouse embryos between E9.5–10.5. RNA modification in mammals, especially in the context of orofacial development, remains an emerging field of research but may prove to be important for our understanding of OFC etiologies.

2.1.2. Teratogen-mediated changes in DNA methylation—The role of DNA methylation in orofacial developmental was initially demonstrated in chemical studies on rodents (Rogers et al., 1994). We therefore start our review of epigenetic studies in the context of teratogen exposure. Application of a global demethylating agent resulted in cleft palate, signifying the importance of the epigenome in orofacial development. Since then, studies have examined methylation dynamics in the *cis*-regulatory elements of specific genes of interest or globally across genomes. Here we examine what is known about DNA methylation changes in teratogen-induced OFCs using animal models. The dependence of orofacial development on the methylation status of a particular gene has yet to be conclusively demonstrated; however, these studies illustrate potential epigenetic mechanisms of OFC etiology imparted solely by environmental factors. In the mouse model, the following three chemicals described in detail cause cleft palate but do not appear to alter lip development.

5'-aza-2'-deoxycytidine: The DNMT-inhibiting chemical 5'-aza-2'-deoxycytidine (AzaD), also known as decitabine, is an azanucleoside that facilitates DNA demethylation. While it is currently being considered for use as a cytostatic agent in cancer therapy (Stresemann & Lyko, 2008; Yu et al., 2018), it has also been used to study the role of DNA methylation during embryogenesis. Exposure of pregnant mice to AzaD resulted in embryos presenting with CPO along with a host of other developmental defects such as hindlimb phocomelia and aberrant rib count (Branch, Chernoff, Brownie, & Francis, 1999; Bulut, Özdemir, Basimoglu-Koca, Korkmaz, & Atalay, 1999; Rogers et al., 1994). The first study associating DNA methylation with palatogenesis observed perturbation of cell cycle phase distribution in mouse embryos maternally exposed to AzaD (Rogers et al., 1994). There was an increased proportion of cells in the S and G2/M phases along with observed cell death in the limb buds and mantle layer of the neural tube. Using an analogous chemical called azacytidine, Bulut et al. (1999) found the window of sensitivity for cleft palate to be embryonic days (E) 11–14

in mice (Bulut et al., 1999). Appropriate global methylation dynamics during this embryonic period are therefore critical to palatogenesis.

More recently, the teratogenic mechanisms of AzaD on palatogenesis were further elucidated by two companion studies. The first study compared transcriptional profiles of BA1 tissue at E9.5 between embryos maternally exposed to AzaD and saline vehicle (Mukhopadhyay et al., 2017). Molecular signaling pathways and transcription factors associated with palatogenesis and cleft palate were differentially expressed in BA1 tissue of the AzaD treatment. However, CpG methylation analysis of select genes revealed that these observed changes in gene expression were likely not due to direct methylation changes at their respective loci. This was confirmed in the companion study utilizing MethylCap-Seq (MCS) analysis in a similar experimental design, although hypomethylation of genes associated with cleft palate was observed, including *Axin2*, *Efna5*, and *Hic2* (Seelan et al., 2017). Interestingly, despite these findings, the latter study identified several differentially methylated regions (DMRs) within endogenous viral elements. The authors postulated that AzaD demethylated these elements and activated endogenous viral transcription. This led the authors to the intriguing proposition that such activity may trigger an interferon-mediated response, thereby leading to downstream changes in the expression of genes involved in palatogenesis. Further investigation is required to validate this hypothesis. Taken together, these two studies demonstrated that aberrant DNA methylation dynamics can perturb developmental gene expression. This can be done without directly impacting the methylation of their respective *cis*-regulatory elements.

Retinoic acid: Other chemicals have the capacity to alter the DNA methylome during orofacial development through less direct mechanisms than AzaD. Retinoic acids (RA) are a group of vitamin A metabolites with an important endogenous role in developmental patterning and fate specification (Rhinn & Dolle, 2012). Nuclear receptors for RA are transcriptional repressors that switch to activators when RA is bound. Retinoids like isotretinoin are also the active ingredient in certain medications such as acne skin creams and cancer therapeutics (Forbat, Ali, & Al-Niaimi, 2018). Developmental exposure to exogenous RA can cause cleft palate in rodents and humans (Ackermans, Zhou, Carels, Wagener, & Von den Hoff, 2011). Dominant negative mutation of retinoic acid receptor alpha (*Rara*) causes cleft palate in mice, indicating that palatogenesis depends on balanced RA signaling (Damm, Heyman, Umesono, & Evans, 1993). During rodent development, exogenous RA exposure using all-trans retinoic acid produces cleft palate with high penetrance, making it an ideal approach to generate chemically induced cleft palate models. Exogenous retinoids (including high doses of vitamin A) appear to disrupt many stages of palatogenesis including palatal shelf outgrowth, elevation, and fusion.

Kuriyama et al. (2008) first identified DMRs in developing mouse secondary palates following maternal exposure to all-trans RA using cytosine extension assay and restriction landmark genomic scanning (Kuriyama et al., 2008). This study found that at E14.5, during palatal fusion, methylation was reduced in CpG islands and in global DNA in the all-trans RA-treatment. In another study, palatal mesenchyme cells (PMCs) of mouse embryos that were maternally exposed to all-trans RA had differential promoter methylation of the transforming growth factor beta (Tgf β) ligand, *Tgfb3* (X. Liu et al., 2016). This was

associated with an increased expression of *Tgfb3* at the transcriptional and translational levels. However, decreased abundance of receptor-regulated Smads along with an increase of inhibitory Smad7 indicated reduced transduction of canonical Tgf β signaling in the all-trans RA treatment. The authors speculated that this was due to an all-trans RA-mediated increase in Tgf β -induced factor homeobox 1 (Tgif1) abundance.

In 2018, two companion genome-wide methylation profile studies were sequentially published that sought to elucidate DNA methylation dynamics during palatal fusion in mice (X. Shu, S. Shu, H. Cheng, et al., 2018; X. Shu, S. Shu, Y. Zhai, et al., 2018). In both studies, maternal exposure to exogenous RA took place at E10.5. Embryonic palates were collected at E14.5 and subject to analysis using a MethylRAD-seq approach that examined methylation at CCGG and CCWGG sites. These studies identified millions of DMRs that were further queried for location relative to genes previously associated with cleft palate. The first study found DMRs in CCWGG motifs within enhancers of three genes: *Hdac4*, a class II histone deacetylase involved in skeletogenesis; *Tcf7l2*, a LEF family transcription factor and Wnt signaling transducer; and *Pdgfrb*, a receptor tyrosine kinase for platelet-derived growth factor (Pdgf) (X. Shu, S. Shu, H. Cheng, et al., 2018). While the enhancer of *Hdac4* was hypermethylated, those for *Tcf7l2* and *Pdgfrb* were hypomethylated. A follow-up study correlated hypermethylation of *Hdac4* with decreased gene transcription (Xuan Shu et al., 2018). The second genome-wide study followed a similar design and identified DMRs associated with three genes that were previously associated with cleft palate: *Hdac4*, as in the previous study; *Smad3*, an activating ligand for Tgf β signaling; and *Mid1*, which functions as an E3-ubiquitin ligase that may affect microtubule polymerization during palatal fusion (X. Shu, S. Shu, Y. Zhai, et al., 2018). In the all-trans RA treatment, *Hdac4* and *Smad3* had increased enhancer methylation while *Mid1* had increased promoter methylation, each of which correlated with decreased expression of the respective gene. While the *Hdac4* methylation localized within CCWGG, the *Smad3* and *Mid1* methylations were present at CCGG motifs within intronic enhancers.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin: Widely considered the most toxic anthropogenic substance, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a halogenated aromatic hydrocarbon that causes cleft palate with high penetrance in developing mice and *ex vivo* human palates (Abbott & Birnbaum, 1991). TCDD and other dioxins are persistent organic pollutants that form as the byproducts of industrial processes such as waste incineration, metal production, paper bleaching, fossil fuel combustion, and the synthesis of chlorophenoxyacetic acid herbicides such as those found in Agent Orange (Courtney & Moore, 1971; Czuczwa & Hites, 1984; Jansson & Voog, 1989; Kunstadter, 1982; C. C. Lee, Shih, & Chen, 2009; Parzefall, 2002; Sweeney & Mocarelli, 2000; Thacker, Nitnaware, Das, & Devotta, 2007). Specifically, TCDD appears to disrupt palatal growth and results in a post-fusional split of the palate in mice (Yamada et al., 2014). An initial study on the epigenetic effect of TCDD exposure investigated promoter methylation of *Tgfb3* in the palates of TCDD-exposed mouse embryos but failed to observe any significant changes during E13.5–15.5 (Pu, Liu, Gan, He, & Fu, 2011). However, Wang et al. (2016) demonstrated that TCDD may impart teratogenicity through increased DNA methylation (C. Wang, Yuan, Fu, & Zhai, 2016). This aberrant hypermethylation appears to occur on E13.5,

while it decreased on E14.5 and 16.5. TCDD-induced global hypermethylation has been correlated with increased transcription of *Dnmt3a*, a DNMT enzyme that facilitates DNA methylation. This increased expression was correlated with CpG hypomethylation in its own promoter. Another research group demonstrated that genes encoding both DNMT and methyl-CpG binding proteins increased expression, adding further evidence that TCDD-mediated cleft palate may be mediated through epigenetic mechanisms (Zhang et al., 2018).

Mouse models have demonstrated that the teratogenic activity of TCDD is dependent on its binding with the aryl hydrocarbon receptor (AHR), a promiscuous nuclear receptor that activates transcription of genes encoding phase I and II xenobiotic-metabolizing enzymes among many others (Fernandez-Salguero, Hilbert, Rudikoff, Ward, & Gonzalez, 1996; Mimura et al., 1997; Peters et al., 1999). It is therefore expected that AHR activation is the upstream transcriptional event that mediates the epigenetic changes described above. Although TCDD studies utilizing AHR knockout mice have been conducted (Mimura et al., 1997; Peters et al., 1999), this has not been investigated in the context of epigenetic changes during palatogenesis.

Cigarette smoke and other chemicals: Cigarette smoke, a known etiological factor of OFCs (Health & Services, 2014), caused C-hypomethylation (5mC) in cultured murine cells from BA1 by increasing the proteolysis of Dnmt1, Dnmt3A, Dnmt3B, and CpG binding proteins (Mukhopadhyay, Greene, & Pisano, 2015). Further, as the product of combusted organic matter, cigarette smoke contains teratogenic ligands of the AHR called polycyclic aromatic hydrocarbons (PAHs). Occupational, periconceptual exposure of women to PAHs has been linked with CL/P (Langlois et al., 2013). It remains to be experimentally determined whether PAHs can induce OFCs through the AHR or epigenetic mechanisms.

We note that other chemicals with OFC hazard are also known to alter C-methylation, including glucocorticoids, heavy metals, and alcohol (Burg, Chai, Yao, Magee, & Figueiredo, 2016; Dixon et al., 2011; R. M. Greene & Pisano, 2010). However, it is unknown whether these can induce OFC through epigenetic changes.

2.1.3. Endogenous DNA methylation dynamics—Outside of teratogen exposures, several studies utilizing the mouse model have advanced our understanding of how differential gene methylation contributes to orofacial development. Similar to the chemical exposure studies, many of these studies are focused on palatogenesis. Kuriyama et al. (2008) noted that CpG methylation between the palates of control animals on E14.5 was significantly elevated compared to E13.5 and E18.5 (Kuriyama et al., 2008). This increased methylation coincides with a critical step in palatogenesis where the shelves are elevated above the tongue just prior to formation of the medial epithelial seam (Ji et al., in this issue).

In 2013, Seelan et al. used a microarray approach to examine global DNA methylation dynamics from E12–14 in mouse palates (Seelan, Appana, et al., 2013). Some key observations were that most of detected genes (73%) were methylated, and while 30% of methylation occurred in CpG islands, most of the methylation occurred within gene bodies (73%) rather than promoters. These observations corroborate all-trans RA studies where DMRs in genes associated with palatogenesis were located at intronic enhancers (X. Shu, S.

Shu, H. Cheng, et al., 2018; X. Shu, S. Shu, Y. Zhai, et al., 2018). Compared to Kuriyama et al. (2008), no DMRs were detected between gestation days at this resolution. However, a more targeted investigation revealed that decreased expression of *Sox4* at E13 and 14 correlated with differential methylation in a CpG-poor promoter region (Seelan, Mukhopadhyay, et al., 2013). *Sox4* is expressed in the medial edge epithelium at E12 but becomes restricted to the epithelial rugae by E14. A target of Tgf β signaling, Sox4 may integrate several developmental signaling pathways that regulate palatal fusion and extension including Wnt/ β -catenin, bone morphogenic protein (Bmp), fibroblast growth factor (Fgf), and Hedgehog (Hh).

Mutant strains have also provided insight toward methylation controls over orofacial development. The A/WySn strain is a mouse line whose offspring have a 15–20% chance of developing CL/P (Plamondon, Harris, Mager, Gagnier, & Juriloff, 2011). The cleft is a multifactorial defect involving an epistatic relationship between three factors: *Clf1*, an intracisternal A particle (or IAP, also an endogenous retroviral transposon) at the 3' end of the *Wnt9b*; *Clf2*, a less-defined locus; as well as a maternal effect. Clf2 appears to suppress the *Clf1* IAP through DNA methylation (Plamondon et al., 2011). It was later found that *Clf1* is a metastable epiallele—that is, its DNA methylation is stochastic during embryogenesis (Juriloff, Harris, Mager, & Gagnier, 2014). Some individuals are consequently deficient in *Clf1* DNA methylation, leaving them susceptible to CL/P mediated by IAP transcription. This implicated metastable epialleles as etiological factors of CL/P.

2.1.4. Human epigenome-wide association studies—In the past decade, methylation profiling of children has implicated epigenetic modification to genes as a possible etiology for OFCs. These modifications are particularly attractive as candidates that contribute mechanistically to environmental etiologies of OFCs. As an example, maternal smoking was found to differentially methylate genes previously associated with OFCs in their children, including *MSX1*, *PDGFRA*, *GRHL3*, *ZIC2*, and *HOXA2* (Joubert et al., 2016). Other methylation profiling studies with patients have identified genes whose variable methylation might contribute to OFC incidence. Table 1 lists human epigenome-wide association studies that examined the methylation profiles of CL/P and CPO patients. Some gene candidates with variable methylation are implicated in OFCs and include transcription factors (*LHX8*, *PRDM16*, *PBX1*, *GSC*, *VAX1*, *MYC*), growth factors and their modulators (*WNT9B*, *BMP4*, *EPHB2*, *BICC1*, *DHRS2*), extracellular matrix genes (*CRISPLD2*, *NTN1*, *CDH1*), and microRNAs (*MIR140* and *MIR300*) (Alvizi et al., 2017; Gonseth et al., 2019; Howe et al., 2019; Z. Xu, Lie, Wilcox, Saugstad, & Taylor, 2019; Zhao, Huang, Zhang, Tang, & Zhang, 2019). Some of the genes listed in Table 1, including *PRDM16* (Pinheiro et al., 2012), *BHMT2* (Zhu et al., 2005), and *WHSC1* (Kim et al., 2008) encode proteins that are involved in methyltransferase activity themselves. Further, studies by Xu et al (2019) and Sharp et al. (2017) investigated how methylation variable positions differ between OFC subsets; the latter investigation identified hundreds of methylation variable positions associated with genes that distinguished between cleft lip with cleft palate (CLP), cleft lip only (CLO), and CPO (Sharp et al., 2017; Z. Xu et al., 2019). DNA methylation profiling appears to be a promising approach and may increase the resolution at which we are able to study etiologies of OFCs.

2.2. Histone modification

DNA is wrapped around histone proteins *in vivo*, and together they make up the majority of chromatin. Post-translational modifications (PTMs) to histone proteins regulate the expression of genes on the histone-associated DNA by altering in accessibility by *trans*-regulatory factors (Suganuma & Workman, 2011). Modifications may also facilitate the recruitment and activation of chromatin remodeling complexes (Jambhekar et al., 2019). The mechanistic details by which this is accomplished are described in several reviews (Jambhekar et al., 2019; Suganuma & Workman, 2011; Zentner & Henikoff, 2013). Aside from DNA methylation, histone methylation and acetylation are perhaps the best understood epigenetic modifications (Bartova, Krejci, Harnicarova, Galiova, & Kozubek, 2008). While histone methylation occurs on arginine (R) and lysine (K) residues, acetylation occurs only on K residues. Other known histone PTMs include biotinylation, phosphorylation, ubiquitination, sumoylation, citrullination, ADP ribosylation, and many others (Latham & Dent, 2007). These modifications, as well as the crosstalk between them, can make the epigenetics of histone modifications quite complex (Nightingale et al., 2007).

Neural crest cells (NCCs) require histone modification at enhancer regions for appropriate chromatin structure and gene expression. As an example, the chromatin of human NCCs rely on H3K4me1 and H3K27ac modifications at the enhancers of genes related to neural crest identity and behavior (Rada-Iglesias et al., 2012). Because of this, the epigenetic mechanisms of DNA methylation and histone modifications can be intrinsically linked. Environmental factors can also influence histone modifications—TCDD exposure, for example, alters the acetylation of H3 during murine palatogenesis (Cuiping et al., 2014). Since histone-modifying enzymes can alter chromatin at several loci, mutations affecting their function often result in syndromes including those that exhibit OFCs (Reynolds et al., in this issue). Here we briefly summarize some mechanistic studies providing evidence for the role of histone modifiers in OFCs. Studies examining histone modifiers in the context of craniofacial structure development or neural crest development are listed in Table 2.

Histone H3K27me3 demethylase KDM6A—Kabuki syndrome is a neurocristopathy that results from mutation affecting either *KDM6A* or *KMT2D*. The former, also called *UTX*, encodes an X-linked H3K27me demethylase. Haploinsufficiency of *KDM6A* was observed in a single patient who presented with multiple developmental abnormalities including stunted development and cleft palate (Lindgren et al., 2013). Nine other patients were identified with similar genotype, but not all exhibited OFCs. The authors followed up with a zebrafish study whereby morpholino knockdown of *kdm6a* expression resulted in disrupted craniofacial development. They concluded that *KDM6A* haploinsufficiency is sufficient to generate cleft palate. A separate zebrafish study likewise identified hypoplastic craniofacial features in *kdm6a* morphants (Van Laarhoven et al., 2015).

Conditional NCC knockout of *Kdm6a* by *Wnt1-Cre* in mice revealed sex specific effects, including cleft palate with incomplete penetrance. Females exhibited greater phenotypic severity than males, possibly because males could compensate with a Y-specific homolog that lacks demethylase activity (Shpargel, Starmer, Wang, Ge, & Magnuson, 2017). This suggested a functional redundancy in males involving a mechanism independent of histone

demethylation. While *Kdm6a* is required for normal chromatin structure and molecular signaling of neural crest cells, the regulated genes did not exhibit aberrant H3K27 or H3K4 trimethylation. Amelioration of the female phenotype with a demethylase-deficient *Kdm6a* knock-in suggested that this protein regulates development independently of its demethylase activity. The lack of altered H3K4 methylation indicated that this may be independent of its methyltransferase properties as well.

Histone H3 lysine 4 methyltransferase KMT2D—Like *KDM6A*, mutations in *KMT2D* are linked to Kabuki syndrome. A study utilized the *Xenopus* model and demonstrated that morpholino knockdown of *kmt2d* presents similar to Kabuki syndrome, a condition involving *KMT2D* mutations that present with cleft palate (Schwenty-Lara, Nehl, & Borchers, 2020). They found that *kmt2d* was required specifically for NCC dispersion but not other migratory behaviors. *kmt2d* morphants exhibited decreased monomethylation of H3K4, confirming a role for histone methyltransferase activity. A likely target gene of *Kmt2d* was *sema3f*, a gene required for cranial NCC migration (Gammill, Gonzalez, & Bronner-Fraser, 2007). Overexpression of *sema3f* partially rescued phenotypes in a gain-of-function experiment.

In contrast, a mouse conditional knockout study did not observe similar effects on NCC migratory behavior (Shpargel, Mangini, Xie, Ge, & Magnuson, 2020). This study did find that, in contrast to conditional *Kdm6A*, conditional *Kmt2d* knockouts by *Wnt1-Cre* had fully penetrant cleft palate. The authors noted that this was consistent with findings in human patients with Kabuki syndrome (Adam et al., 2019). The palate of *Kmt2d* cKO heterozygotes, which exhibited aberrant expression of extracellular matrix components, failed to develop properly and did not elevate.

Downstream of *Kmt2d*, Kabuki syndrome phenotypes may also be caused by *RAP1A* loss of function. This causes inappropriate RAS/MAPK activation and associated structural defects (Bogershausen et al., 2015). A chemical screen using a zebrafish Kabuki syndrome model (CRISPR-mediated *kmt2d* KO) found that small molecule inhibitors of MAPK signaling could prevent Kabuki-related structural defects during embryogenesis (Tsai et al., 2018). One compound, desmethyl-Dabrafenib, was effective at a 100 nM dose with no obvious signs of toxicity. Although the efficacy of such a treatment in mammals or humans is not known, this study serves as a compelling example of how effective use of alternative models could potentially lead to the preventive treatment of some congenital disorders.

Histone-lysine demethylase PHF8—Mutations in proteins that modify histone PTMs, such as K demethylases, can result in loss-of-function alleles with a host of severe downstream effects. *PHF8* encodes a H4K20 and H3K9 demethylase whose mutation results in human cases of CL/P associated with X-linked intellectual disability (Abidi, Miano, Murray, & Schwartz, 2007; Fortschegger et al., 2010; Laumonnier et al., 2005; Loenarz et al., 2010). Its demethylase activity is required for ribosomal RNA (rRNA) gene transcription (Feng, Yonezawa, Ye, Jenuwein, & Grummt, 2010), and it also interacts with *Rara* to promote neural differentiation (Qiu et al., 2010). Interestingly, the catalytic domain of PHF8 is a 2OG oxygenase, which may implicate a hypoxic mechanism for OFC in affected children whose mothers were periconceptionally exposed to tobacco smoke (Loenarz et al.,

2010). Phf8 was demonstrated to additionally regulate expression of the OFC-related ortholog *msx1* in zebrafish (Qi et al., 2010). Thus, *PHF8* may also be important for appropriate neural crest induction, patterning, and survival through regulating *MSX1* (Ishii et al., 2005; Monsoro-Burq, Wang, & Harland, 2005). Additionally, its overexpression in murine bone marrow stromal cells promoted bone regeneration of mouse calvaria, likely through the regulation of *SATB2* (Han et al., 2015). This indicates a probable role for *PHF8* in future regenerative therapeutics for craniofacial defects.

Histone-lysine N-methyltransferase MECOM (PRDM3)—PRDM3 regulates methylation of H3K4 and H3K9 residues. During zebrafish development, *prdm3* is highly expressed in the pharyngeal arches (Ding, Clouthier, & Artinger, 2013). Morpholino knockdown results in neuro- and viscerocranial defects along with decreased expression of the NCC markers *dlx2a* and *barx1*. Another study observed similar effects of *prdm3* knockdown in developing zebrafish, along with decreased methylation of H3K4 and H3K9 (Shull et al., 2020). In developing mice, conditional knockout of *Prdm3* by *Sox2-Cre* results in mid-gestation lethality.

Histone-lysine N-methyltransferase PRDM16—*prdm16* exhibits overlapping function with *prdm3* in developing zebrafish. Similar to the latter gene, *prdm16* knockdown reduces expression of *dlx2a* and *barx1* (Ding et al., 2013) as well as methylation of H3K4 and H3K9 (Shull et al., 2020). Loss of function by N-ethyl-N-nitrosourea mutagenesis (Bjork, Turbe-Doan, Prysak, Herron, & Beier, 2010), RNA interference (Bjork, Fujiwara, et al., 2010), and gene trap (Strassman et al., 2017) revealed that it is required for palatogenesis in mice. Prdm16 is capable of binding the promoters of genes involved in myogenesis, chondrogenesis, and osteogenesis in cells of the developing mouse palate (D. R. Warner, Mukhopadhyay, Webb, Greene, & Pisano, 2012); expectedly, null mice exhibit increased expression of genes related to Tgf β and Bmp signaling, as well as differential expression of genes involved in chondrogenesis and osteogenesis (D. R. Warner, Wells, Greene, & Pisano, 2013). In conditional knockouts (*Sox2-Cre*), Prdm16 was found to regulate H3K9 but not H3K4 methylation (Shull et al., 2020). In addition to its activity toward histones, Prdm16 may regulate orofacial development through its activities toward Smad transcription factors (D. R. Warner et al., 2007).

Arginine methyltransferase PRMT1—*PRMT1* encodes an arginine methyltransferase that targets H4R3 to ultimately form a H4R3me2a modification. It also regulates greater than 85% of arginine methyltransferase activity in human cells (Tang et al., 2000); however, some of this activity may be directed toward non-histone substrates (J. Xu et al., 2013). Conditional knockout of mouse *Prmt1* in NCCs by *Wnt1-Cre* causes several craniofacial skeletal malformations as well as cleft palate (Gou, Li, Wu, et al., 2018). These deformities were similar to those observed in *Msx1*-null mice (Satokata & Maas, 1994). *In situ* hybridization confirmed that *Prmt1* cKO decreased expression of *Msx1* in the frontal bone primordium and mandible at E12.5, a stage where *Msx1* should be highly expressed in the mouse embryo head. A follow-up study found that Bmp signaling was disrupted during conditional knockout of *Prmt1* (Gou, Li, Jackson-Weaver, et al., 2018), which was consistent with its methyltransferase activity directed toward the Bmp-inhibitory Smad6 (J. Xu et al.,

2013). However, H4R3me2a was decreased in conditional knockouts, indicating that some of its effect on development may be through histone modification.

Histone methyltransferase WHSC1—The methyltransferase gene *Whsc1*, whose loss of function is associated with Wolf-Hirschhorn syndrome, is expressed in palatal epithelium and mesenchyme throughout murine palatogenesis (S. Liu, Higashihori, Yahiro, & Moriyama, 2015). Its expression in the developing palate was reduced following administration of all-trans RA to pregnant females at levels sufficient to induce cleft palate. The authors of this study speculated that *Whsc1* was involved in promoting cell proliferation. In another study, knockdown of *whsc1* during *Xenopus* development resulted in decreased facial width and midfacial area. There was also a decrease in the total area of cranial NCC streams, which had reduced migratory distance (Mills et al., 2019).

Histone deacetylases HDAC3 and HDAC4—The histone deacetylase gene *Hdac3* is required for life in developing mice, and conditional knockout in NCCs (*Wnt1-Cre*) results in several craniofacial defects including cleft palate (Singh et al., 2013). A major role for *Hdac3* in NCCs appears to be regulation of the transcription factors *Msx1*, *Msx2*, and *Bmp4*. In conditional knockouts, developing mice had increased expression of these genes coinciding with decreased cell proliferation and apoptosis at E12.5. Although histone modifications were not directly measured, acetylation is likely an important mechanism for proper balance of gene expression in NCCs.

The class II histone deacetylase HDAC4 is involved in osteogenesis at least in part through its interactions with the transcription factor MEF2, allowing it to regulate endochondral ossification (Arnold et al., 2007). In zebrafish development, *hdac4* is expressed in pre-migratory as well as migrating cranial NCCs (DeLaurier et al., 2012). Morpholino knockdown either reduced or abolished cranial NCCs and resulted in palatal defects such as a shortened, clefted, or missing ethmoid plate.

Histone acetyltransferase KAT6A—Microdeletion or mutation of *TBX1* in humans leads to DiGeorge Syndrome, characterized by submucous cleft palate (SMCP), heart defects, thymic dysfunction, among other symptoms (Voss et al., 2012). In mice, homozygous deletion of the acetyltransferase *Kat6a*, which is involved in *Tbx1* expression, resulted in a partial phenocopy of DiGeorge syndrome including SMCP. The authors showed that a single additional copy of *Tbx1* was not sufficient to rescue the palatal effects of *Kat6a* deficiency. This indicated that either a greater dosage of the *Tbx1* gene product was necessary, or *Kat6a* may act on other genes in addition to *Tbx1*.

2.3. Folate metabolism, epigenetics, and orofacial clefts

Folate metabolism is an important process during embryogenesis. A recent systematic review and meta-analysis found an inverse association between folic acid supplementation and OFC, although this conclusion might be affected by heterogeneity between studies, incomplete reporting of population characteristics, and variation in timing of exposure and supplement types, which are common in such analyses (Zhou et al., in this issue). As previously mentioned, methyltransferases rely on products of the folate cycle to receive or

donate methyl groups to intermediate carrier molecules (Seelan et al., 2019). This involves the transfer of methyl groups from folate to SAM through a series of enzymatic reactions. DNMTs transfer the methyl group from SAM to cytosine, while histone methyltransferases transfer methyl from SAM to K or R histone residues. There is evidence that folate supplementation protects against birth defects associated with aberrant neural tube and craniofacial development, such as variants of spina bifida (Williams et al., 2002) and OFCs (Wilcox et al., 2007). The exact mechanism by which folate protects orofacial development, however, remains unclear. It has been hypothesized that the protective effects of folate during neural tube closure and lip/palate fusion are related at least in part to facilitating DNA and histone methylation patterns during development. Indeed, a mutation impacting a folate cycle enzyme was found to transgenerationally affect development and induce epigenetic destabilization in mice (Padmanabhan et al., 2013). Alternatively, since folate is required for DNA synthesis, the competing hypothesis is that folate is protective against OFCs by promoting cell proliferation (Wahl et al., 2015).

The *Xenopus* model has been particularly useful for investigating the connection between folate metabolism, orofacial development, and DNA/histone methylation. In *Xenopus*, the reduced folate carrier (Xrfc) is a transporter that moves folate into the cell where it undergoes metabolism in the folate cycle. Morpholino knockdown of Xrfc, which was normally expressed abundantly in the craniofacial region, caused craniofacial defects in tadpoles and reduced expression of the NCC markers *zic1*, *snail2*, and *foxd3* (J. Li, Shi, Sun, Zhang, & Mao, 2011). Normal development and expression of NCC markers was restored with 5-methyltetrahydrofolate, indicating the effects were related to folate metabolism. Further, animal cap assays demonstrated that Xrfc knockdown decreased H3K4 mono- and tri-methylation but could be rescued by 5-methyltetrahydrofolate. Thus, folate transport from the environment into the cell is a requirement for appropriate histone methylation. Another *Xenopus* study found that knockdown of dihydrofolate reductase, a step in the folate cycle preceding SAM synthesis, resulted in craniofacial hypoplasia (Wahl et al., 2015). While reduced cell proliferation and increased cell death were observed, significant changes in global DNA methylation or H3K4 trimethylation were not. The authors noted that their study cannot rule out the possibility that methylation of DNA or histones was altered, but it does strongly support the hypothesis that folate supports cell proliferation and survival during craniofacial development.

Human studies have also supported the role of dietary folate in epigenetic-mediated CL/P. The epigenome-wide association study conducted by Gonseth et al. (2019) specifically examined correlations between epialleles and OFCs in the U.S. population prior to when the mandate of dietary folate fortification was fully implemented in 1998 (Gonseth et al., 2019). Consistent with the role of folate in epigenetics, global DNA status in this population skewed toward hypomethylation with DMRs present on genes previously associated with CL/P (see Table 1). This supported the hypothesis that folate deficiency contributes to OFC through changes in DNA methylation, but it does not rule out the possibility of other mechanisms.

Additional studies on the role of folate supplementation, as well as manipulations of the folate cycle, will need to be undertaken to better understand its role in epigenetic changes

during orofacial development. Epigenome-wide association studies have laid the groundwork for mechanistic studies in animal models. More information on the role of the folate cycle outside of an epigenetic context is described in a companion review (Garland et al., 2020b, in this issue).

3. MicroRNAs and orofacial clefts

Attention has increasingly focused on characterizing the role of non-coding RNA (ncRNA) in mediating gene expression during developmental and pathophysiological processes. Here we will discuss the emerging functional and diagnostic roles of ncRNA in OFCs with a focus on microRNA (miRNA).

Since their discovery in the early 1990s, miRNAs have emerged as the largest and best-studied class of ncRNAs with indispensable roles in regulating animal development (C. T. Lee, Risom, & Strauss, 2006). In humans, miRNAs are estimated to regulate the translational expression of 60% of protein-coding genes (Friedman, Farh, Burge, & Bartel, 2009), and their misexpression is implicated in various pathologies including OFCs (A. Suzuki et al., 2018). They are a class of small RNAs, ranging from 18 to 25 nucleotides in length, that can antagonize gene expression through a variety of post-transcriptional mechanisms involving recruitment of the RNA-induced silencing complex (RISC) to a target sequence. Notably, miRNA activity can exhibit both multiplicity, where one miRNA can target multiple mRNAs, and redundancy, where multiple miRNAs can target one mRNA (Catalanotto, Cogoni, & Zardo, 2016). We will briefly summarize miRNA biogenesis and mechanisms of action, and then discuss our current understanding of how miRNA-related dysfunction is involved in OFCs.

3.1. miRNA mechanisms of action

Despite their size, miRNAs are quite complex in terms of their structural and functional diversity. Therefore, in studies that determine the global importance of miRNAs for any given biological process, the details of miRNA biogenesis are important to consider. miRNAs undergo several processing steps from initial transcription to maturity (Faller & Guo, 2008). Canonically, they are first transcribed as long primary miRNA (pri-miRNA) hairpins. Some pri-miRNAs are polycistronic, whereby multiple miRNAs are encoded within one primary transcript (Tanzer & Stadler, 2004). They are then trimmed into 70 base pair (bp) precursors (pre-miRNAs) by the microprocessing complex, which consists of Drosha and DiGeorge syndrome chromosomal region 8 (DGCR8) (Catalanotto et al., 2016). pre-miRNAs are exported across the nuclear membrane by the Exportin5-Ran-GTP complex to the cytoplasm. Next, an RNase called Dicer cleaves pre-miRNA into an 18–25 bp duplex consisting of two complementary mature miRNAs that are referred to as -5p and -3p (previously miR*) strands. The duplex is unwound and either the -5p or -3p strand is loaded onto Argonaute (AGO), the key protein constituent of RISC. Thermodynamics determine which strand is preferentially loaded. Once the mature miRNA is loaded onto AGO, RISC typically binds the 3'-untranslated region (UTR) of target mRNA through Watson-Crick base pair complementarity of the guiding miRNA. The efficiency of RISC in mediating post-transcriptional repression depends on the degree of complementarity between the 5' seed

region of the miRNA (nucleotides 2–8) and the 3'-UTR. For example, perfect complementarity can result in direct cleavage by AGO. Less-than-perfect complementarity may result in destabilization of mRNA through an increased rate of deadenylation of the poly-A tail. If a target mRNA reaches the ribosome, RISC may cause steric hindrance and prevent translation (Catalanotto et al., 2016).

miRNAs exhibit a wide range of functional diversity. In some cases, either the -5p and -3p strand can serve as a functional guide, thereby increasing the diversity of targets (Catalanotto et al., 2016). In addition, alternate processing mechanisms can affect miRNA biogenesis. Some miRNAs are transcribed as pre-miRNAs that do not undergo Drosha processing. These transcripts are capped with 7-methylguanosine, which creates a bias toward -3p strands as guides (Xie et al., 2013). Some miRNAs exist within the introns of protein-coding genes and are referred to as miRtrons. These are spliced out by the spliceosome and do not undergo Drosha processing (Berezikov, Chung, Willis, Cuppen, & Lai, 2007). Further, the adenosine deaminase ADAR can convert adenosine (A) to inosine (I), which preferentially binds with cytosine (C) rather than uracil (U). Unstable A-U mismatches can prevent processing by either Drosha or Dicer depending on the location, and modifications to the seed region can shift the function of miRNAs toward an alternate set of targets (Faller & Guo, 2008).

3.2. Role of global miRNA expression in orofacial clefts

Global expression studies using microarrays or small RNA sequencing have implicated over 100 specific miRNAs in mammalian orofacial development. In a study examining global expression of 588 miRNAs in mice from embryonic days (E)12–14, over 26% were detected during orofacial development across the measured time points (Mukhopadhyay et al., 2010). The authors also found that differentially expressed miRNAs were predicted to target genes involved in several processes required for normal orofacial development, including cell migration, adhesion, differentiation, apoptosis, and epithelial-mesenchymal transition (EMT). Another mouse study on miRNA expression in the upper lip during E10–11.5 revealed differential expression of 142 miRNAs. The five having the greatest increased expression belonged to the let-7 family, while those with the greatest decreased expression belonged to the miR-302/367 family. This study also found that differentially expressed miRNAs were predicted to target expression of key genes during lip development including the tumor suppressor *p63* (D. R. Warner et al., 2014). Using *Tgfb3* null mice, a separate study found that miRNAs involved in apoptosis (miR-206 and miR-186) are differentially regulated by the Tgf β pathway in medial edge epithelium cells (D. Warner et al., 2015). miRNA gene methylation may also play a role in palatogenesis, as miRNAs derived from both methylated and unmethylated DNA were predicted to target genes related to pathways involved in palate development (Seelan et al., 2014). As previously mentioned, two recent epigenome-wide association studies identified miR-140 and miR-300 as DMRs associated with OFCs. miRNA dysfunction can therefore potentially impact several critical processes during orofacial development through a variety of means.

Removal of *Dicer* is sufficient to prevent expression of functional miRNAs, and its deletion is the choice approach for studying the role of global miRNA expression (Graves & Zeng,

2012). Since complete homozygous knockout (KO) of *Dicer* is lethal to mouse embryos shortly after implantation, conditional KOs have been utilized to study the tissue-specific importance of miRNA expression during craniofacial development (Schoen et al., 2017). This approach has been particularly useful for characterizing the specific role of miRNAs in mesenchymal and epithelial tissues. Conditional KO of *Dicer* in cranial neural crest-derived mesenchyme, mediated through *Wnt1-Cre*, resulted in cleft palate along with extreme craniofacial abnormalities and perinatal mortality (Oommen et al., 2012). Using *Pitx2-Cre*, conditional KO of *Dicer* in the oral ectoderm resulted in dental abnormalities with incomplete penetrance of cleft palate (Cao et al., 2010). Taken together, these two previous studies demonstrated that miRNA-related dysfunction in either mesenchymal or epithelial tissue could lead to cleft palate.

3.3. Role of specific miRNAs in orofacial clefts

Once a global role for miRNAs in craniofacial development had been established, focus shifted toward identifying individual miRNAs (or miRNA clusters) that are associated with OFCs. Several different approaches have been taken to identify miRNAs associated with craniofacial development. Although over a hundred miRNAs have been implicated in craniofacial development, we focus primarily on those whose association with OFCs (or genes involved in OFCs) have been validated either *in vivo* or through *in vitro* methods, or by human genetic studies. Here we describe how a selection of these studies have started to dissect the role of miRNAs in OFCs. A list of mechanistic studies in animal models or cell lines is provided in Table 3, while a list of human gene-OFC association studies is provided in Table 4.

Gene encoding ligands, receptors, modulators, and transducers for major developmental signaling pathways are targeted by miRNAs during orofacial development. The first experiment to mechanistically demonstrate this was a zebrafish study published by (Eberhart et al., 2008). Injection of miR-140 duplex was found to disrupt palatogenesis in embryos by modulating expression of the Pdgf receptor, *pdgfra*, leading to cleft in palatal cartilage. Shortly after, a human case-control study discovered a significant correlation between a single nucleotide polymorphism (SNP) in miR-140 and non-syndromic cleft palate (NSCP) (L. Li, Meng, Jia, Zhu, & Shi, 2010). This mutation (rs7205289) resulted in a C to A transversion in the pri-miRNA, which was predicted to create an RNA bulge at the Drosha processing site. Using transfected HEK293 cells, the mutant allele was found to increase the abundance of miR-140-3p while decreasing that of miR-140-5p. A follow-up study validated *Pdgfra* as a miR-140 target in murine PMCs. A role for *PDGFRA* regulation by miR-140, in association with human CPO, was confirmed shortly afterward (Rattanasopha et al., 2012). More recently, miR-140 was also found to regulate expression of a bone morphogenetic protein gene *BMP2* and a fibroblast growth factor gene *FGF9* in human PMCs (A. Li et al., 2020).

As detailed in a companion review (Ji et al., in this issue), elongation and elevation of the palatal shelves during craniofacial development involves epithelial to mesenchymal transition (EMT) and other processes including apoptosis. These are under the control of several crucial morphogenetic signaling pathways (Reynolds et al., in this issue), including

Tgfb signaling. In mice, miR-200b expression was detected in the palatal mesenchyme and medial edge epithelium prior to shelf fusion, and in the midline epithelial seam during fusion (Shin et al., 2012). It was found to directly regulate expression of *Smad2*, a Tgfb signal transducer, and *Snail*, a regulator of EMT downstream of Tgfb, during mouse palatogenesis. Ectopic overexpression of miR-200b prevented complete fusion of the palates, repressed expression of *Smad2* and *Snail*, and altered cell proliferation and apoptosis. This indicated that miRNA regulation is important for critical signaling pathways during palatogenesis in mammals.

The miR-17-92 cluster, which is expressed in the first branchial arch of mouse embryos during palatogenesis, is a known inhibitor of Tgfb in cancer cells. This led Li et al. (2012) to hypothesize that the miR-17-92 cluster targets Tgfb signaling during palatogenesis. They found that miR-17-92 had decreased expression in PMCs from E12-14 (L. Li, Shi, Zhu, & Shi, 2012). Mimics of individual miRNAs from the miR-17-92 cluster were used for luciferase reporter assays in cultured PMCs. miR-17 and miR-20a, which share identical seed sequences, targeted the Tgfb receptor *Tgfb2*, while miR-18a targeted the Tgfb transducers *Smad2* and *Smad4*. This indicated that reduced expression of miR-17-92 during palatogenesis may facilitate EMT and other processes mediated by Tgfb signaling.

In a separate study, Wang et al. (2013) found miR-17-92 loss-of-function mouse mutants had incompletely penetrant cleft lip and palate along with mandibular hypoplasia (J. Wang et al., 2013). Compound miR-17-92/miR-106a-25 mutants (the latter cluster being a paralog of miR-17-92) had cleft secondary palate coinciding with completely penetrant cleft lip and palate. Thus, functional redundancy between miRNAs or associated clusters may help to explain how some alleles might lead to incomplete penetrance of orofacial clefting. Furthermore, while Li et al. (2012) found a link between miR-17-92 and Tgfb, Wang et al. (2013) identified other miR-17-92 target genes involved in craniofacial development such as *Tbx1*, *Tbx3*, *Fgf10*, *Shox2*, and *Osr2*. They also demonstrated that miR-17-92 was regulated by Bmp signaling and its expression was directly controlled by Ap-2a. This is consistent with previous findings linking deficient BMP signaling to OFCs in both humans and mice (W. Liu et al., 2005; S. Suzuki et al., 2009) (Reynolds et al., in this issue). These results demonstrated two key points for craniofacial development: 1) Bmp signaling may be transduced through both Smad factors and miRNA-17-92; and 2) miR-17-92 may exhibit multiplicity of action by targeting several pathways, including Tgfb, Fgf, Wnt, and others.

Ding et al. (2013) used the zebrafish larval model to identify miR-133b as a miRNA whose overexpression resulted in midfacial cleft (Ding et al., 2016). These findings were corroborated by Suzuki et al. (2019) using human PMCs (A. Suzuki, Li, et al., 2019). Among the validated target genes of miR-133b in human cells were key regulators of palatogenesis, including an Fgf signaling receptor *FGFR1* and the transcription factor *PAX7*. Together, these two studies demonstrated that interspecies comparisons can be made to identify clinically relevant genes involved in OFCs.

Subsequent studies identified multiple OFC-related miRNAs at a time using microarrays or small RNA sequencing, but not all had experimentally validated miRNA targets. Using tissue collected from patients in China, Wang et al. (2017) identified miR-24-3p,

miR-1260b, and miR-205–5p as having significantly increased expression in CL/P patients relative to controls (S. Wang et al., 2017). Although they did not experimentally validate targets, they predicted that these miRNAs targeted genes in the WNT signaling pathway. Schoen et al. (2018) identified three miRNAs (miR-18a-5p, miR-92a-3p, miR-93–5p) that increased expression and six (miR-181a-5p, miR-29c-5p, miR-3182, miR-451a, miR-549a, miR-92b-5p) that decreased expression among CL/P patients (Schoen et al., 2018). They also identified one miRNA (miR-505–3p) that decreased expression among CPO patients.

Two recent studies leveraged the literature and *in silico* modeling to identify miRNAs associated with OFCs and subsequently validate predicted target genes in cultured human cells. Suzuki et al. (2019), in addition to miR-133b, identified miR-374a-5p and miR-4680–3p as being differentially regulated in CPO patients (A. Suzuki, Li, et al., 2019). The latter two miRNAs were validated to target *WNT5A* expression using miRNA mimics in human PMC. Additionally, among other targets, miR-374a-5p was validated to target *MSX1*, while miR-4680–3p was validated to target *ERBB2* and an enzyme involved in folate metabolism, *MTHFD1*. Gajera et al. (2019) utilized a similar approach using data from CL/P patients (Gajera et al., 2019). miR-497–5p and miR-655–3p mimics were able to suppress proliferation in human lip cell cultures. Validated targets for miR-497–5p included *TFAP2A* (*AP-2a*), *FGFR1*, *FOXP2*, and *RUNX2*, while targets for miR-655–3p included the WNT receptor *FZD6*, xenobiotic metabolizing enzyme *CYP1A1*, and the pro-survival factor *BCL2*.

Like gene methylation, mutations in the miRNA binding sites within a given gene's 3'-UTR may also partly account for CLO. Since miRNA expression varies spatiotemporally, their differential abundance may provide a mechanism that explains how some mutations are specific to OFC subtypes. In studies of a Chinese population, mutations within miRNA binding sites in the 3'-UTR of *FGF5* and *FGF2* were significantly associated with CLO (as well as CPO and CL/P, respectively), implicating the Fgf pathway in distinct OFC subtypes (D. Li et al., 2016). The interaction of miR-145 and miR-469 within the binding sites of these respective genes was confirmed in mouse, monkey, and human cells. Another study on the same population identified a mutation in the 3'-UTR of *FOXE1*, a gene associated with OFCs in Caucasian populations, having significant association with CLO (Yin et al., 2016). This mutation was within the binding site of miR-423–3p, and its adverse effect on miRNA binding was confirmed in mouse and monkey cell lines. Further, a study on genes associated with murine cleft lip revealed that some may be regulated by miR-124–3p (A. Suzuki, Yoshioka, et al., 2019). Three genes, *Cdc42*, *Ifi88*, and *Pbx3*, were regulated by miR-124–3p in both murine embryonic lip mesenchymal cells and O9–1 cranial neural crest cells. When miR-124–3p was overexpressed, proliferation was suppressed in both lip mesenchymal cells and O9–1 cells. Taken together, these studies demonstrate that miRNAs likely factors that contribute to the stratification of OFC subtypes.

4. Conclusion

The etiology of OFCs is complex and may often involve gene-environment interactions that are yet to be understood; however, what was once a black box of complicated dysregulation is now being unpacked. Epigenetic changes such as DNA methylation and histone

modifications are key developmental mechanisms having well-established links with the environment. The application of newer technologies and approaches will facilitate the integration of genetic and environmental data, and it is likely that epigenetic and possibly other non-coding mechanisms will be at the forefront of these interactions. To date, relatively few epigenome-wide association studies involving OFC patients have been performed, but they have already contributed significant evidence toward the distinction of OFC subtypes (*e.g.*, cleft lip only). Profiling between OFC subtypes, as well as between OFC-afflicted and control individuals, has identified differential methylation of genes related to WNT, BMP, and ephrin signaling, along with genes involved in metabolism, cellular structure, transcriptional control, and histone methylation. By increasing the resolution at which we can distinguish OFC subtypes, we can better understand the specific mechanisms contributing toward distinct phenotypes.

Dysregulation of miRNA expression, as well as mutations in miRNA seed sequences and the 3' UTR of target genes, are demonstrated factors leading to OFC in humans. Further, these factors may also play a role in development of distinct OFC subtypes. In roughly the past decade, miRNAs have been confirmed to regulate developmental pathways including Pdgf, Tgf β , Bmp, Fgf, Wnt, epidermal growth factor, and others with known roles in craniofacial development. miRNAs also target transcriptional regulations such as T-box factors as well as enzymes involved in the folate cycle. Through these pathways, miRNAs control important processes in orofacial development including cell migration, differentiation, proliferation, apoptosis, and EMT. Although recent advancements will prove useful for understanding OFC etiologies, miRNAs appear to have more complicated mechanisms of regulation. These include the formation of competitive endogenous networks with long non-coding RNAs (lncRNA) (Y. Gao et al., 2019), as well as the ability to bind gene promoters and activate their expression through a process called RNA activation (Ramchandran & Chaluvally-Raghavan, 2017). As in the past decade of research, it is expected that the near future will bring informative advances in our understanding of OFC etiology for both miRNAs and epigenetic processes.

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Figure 1. Simplified diagram of DNA methylation motifs associated with a hypothetical gene. **a)** Double-stranded methylation is understood to occur at CpG and CCWGG motifs (green), where W represents either adenine or thymine. **b)** DNA methylation may occur at the promoter region, which are often enriched with CpG motifs (blue circles). Other gene regulatory regions, such as flanking and intronic enhancers, may also be methylated at both CCWGG (orange circle) and CpG motifs. **c)** A simplified example of how DNA methylation at the promoter is catalyzed by DNA methyltransferases (DNMT) to prevent gene expression. Methylation spatially hinders transcription factor (TF) binding to the promoter by facilitating recruitment of CpG-binding proteins and histone modification.

Table 1.

List of human methylation studies relating methylation variable positions and their nearest genes to non-syndromic OFCs. CLO: cleft lip only; CPO: cleft palate only; CLP: cleft lip with cleft palate; CL/P: cleft lip with or without cleft palate; NSD: no structural defect.

Gene nearest each methylation variable position	Population	Sample size	Tissue(s) analyzed	References
<i>CDH1, MYC, FAT1, WHSC1, MARK1*</i>	Individuals from Brazil and United Kingdom	Brazil: CL/P: n = 67 NSD: n = 59 United Kingdom: CL/P: n = 171 NSD: n = 177	Blood; lip	(Alvizi et al., 2017)
<i>TBX1, COL11A2, CRB2, HOXA2, PDGFRA, CRISPLD2, SMOG1, PRVL1, CCL2</i>	Children with non-syndromic OFC born between 2013–2016 (United Kingdom)	CLO: n = 49 CPO: n = 49 CLP: n = 49	Blood, lip, or palate tissue collected during corrective surgery	(Sharp et al., 2017)
<i>BICCI1, CLASRP</i>	Infants 2–3 days old (Norway)	CLO: n = 92 CPO: n = 84 CLP: n = 132 NSD: n = 436	Blood	(Z. Xu et al., 2019)
<i>RGS14, DLGAP2, BANP, CPQ, MIR300, NINJ2, DAB2IP, AFDN, GRID2IP, FSCN2, CCDC50, RD3, MAPK14, AOA1, CRIL, STEAP1B, MGAT5B, XYLT1, CNST, LGAL58</i>	Individuals from Brazil	CL/P: n = 67 NSD: n = 59	Blood	(Zhao et al., 2019)
<i>VAX1, LOC146880, NTN1</i>	Integration of multiple data sources. DNA methylation profiles derived from (Alvizi et al., 2017) and (Sharp et al., 2017).			(Howe et al., 2019)
<i>VTRNA2-1, MIR140, LHX8, PRDM16, WNT9B, PBX1, CRISPLD2, GSC, BMP4, EPHB2, DHRS2</i>	Newborn live births between 1988–1997 (United States)	CL/P: n = 97 NSD: n = 93	Archived newborn bloodspots	(Gonseth et al., 2019)

Table 2.

List of functional studies demonstrating a relationship between histone modifiers and OFCs.

Gene	Syndrome	Histone activity	References	Species
(a) Histone methylation				
KDM6A	Kabuki	H3K27 demethylase H3K4 methyltransferase	(Lindgren et al., 2013)	Zebrafish
			(Bogershausen et al., 2015)	Zebrafish
			(Shpargel et al., 2017)	Mouse
KTM2D	Kabuki	H3K4 methyltransferase	(Bogershausen et al., 2015)	Zebrafish
			(Tsai et al., 2018)	Zebrafish
			(Schwenty-Lara et al., 2020)	Xenopus
			(Shpargel et al., 2020)	Mouse
PHF8	XLID with CL/P	H4K20 demethylase H3K9 demethylase	(Qi et al., 2010)	Zebrafish
			(Han et al., 2015)	Mouse
PRDM3	-	H3K4 methyltransferase H3K9 methyltransferase	(Ding et al., 2013)	Zebrafish
			(Shull et al., 2020)	Mouse, Zebrafish
PRDM16	-	H3K4 methyltransferase H3K9 methyltransferase	(Bjork, Turbe-Doan, et al., 2010)	Mouse
			(Bjork, Fujiwara, et al., 2010)	Mouse
			(D. R. Warner et al., 2012)	Mouse
			(Ding et al., 2013)	Mouse
			(D. R. Warner et al., 2013)	Muse
			(Strassman et al., 2017)	Mouse
			(Shull et al., 2020)	Mouse, Zebrafish
PRMT1*	-	H4R3 methyltransferase	(Gou, Li, Wu, et al., 2018)	Mouse
			(Gou, Li, Jackson-Weaver, et al., 2018)	Mouse
WHSC1	Wolf-Hirschhorn	H3K36 methyltransferase	(S. Liu et al., 2015)	Mouse
			(Mills et al., 2019)	Xenopus
(b) Histone acetylation				
HDAC3		N-terminal core histone deacetylase	(Singh et al., 2013)	Mouse
HDAC4		N-terminal core histone deacetylase	(DeLaurier et al., 2012)	Zebrafish
KAT6B		H3 acetyltransferase	(Voss et al., 2012)	Mouse

Table 3.

List of individual miRNAs or miRNA clusters associated with OFCs. miRNAs were selected from animal, organ, and cell studies that demonstrated a relationship with OFCs. miRNA targets include only those that were validated by anti-correlated expression analysis or reporter assays. CL/P, cleft lip with or without cleft palate; CLO, cleft lip only; CPO, cleft palate only; LMCs, lip mesenchymal cells; MFC, medial facial cleft; OFC, orofacial cleft; PMCs, palatal mesenchyme cells.

miRNA	Target genes	Models	OFC subtype	References
miR-140	<i>PDGFRA</i>	Zebrafish	CPO	(Eberhart et al., 2008)
	-	Human HEK293 cells	-	(L. Li et al., 2010)
	<i>PDGFRA</i>	Mouse PMCs	-	(L. Li et al., 2011)
	<i>PDGFRA</i>	Monkey COS7 cells	-	(Rattanasopha et al., 2012)
	<i>BMP2, FGF9</i>	Human PMCs	-	(A. Li et al., 2020)
	<i>FGF9, PDGFRA</i>	Mouse PMCs	-	
miR-200b	<i>SMAD2, SNAIL</i>	Mouse; mouse cultured palates; human HEK293 cells	CPO	(Shin et al., 2012)
miR-17-92* (cluster)	<i>TGFBR2, SMAD2, SMAD4</i>	Mouse PMCs	-	(L. Li et al., 2012)
	<i>TBX1, TBX3, FGF10, SHOX2, OSR1</i>	Mouse	CL/P	(J. Wang et al., 2013)
	<i>E2F1</i>	Mouse PMCs	CL/P	(L. Li et al., 2017)
	<i>TGFBR1, TGFBR2</i>	Mouse	CL/P	(Ries, Yu, Holton, Cao, & Amendt, 2017)
miR-3649	<i>MSX1</i>	Human HEK293A cells; Monkey COS7 cells	OFC	(L. Ma et al., 2014)
miR-96	<i>TBX1</i>	Mouse	CPO	(S. Gao et al., 2015)
miR-145	<i>FGF5</i>	Human HEK293A cells; Monkey COS7 cells; Mouse C2C12 cells	OFC	(D. Li et al., 2016)
miR-187	<i>FGF9</i>			
miR-469	<i>FGF2</i>			
miR-423-3p	<i>FOXE1</i>	Monkey COS7 cells; Mouse C2C12 cells	CLO	(Yin et al., 2016)
miR-133b	-	Zebrafish	MFC	(Ding et al., 2016)
	<i>FGFR1, GCH1, PAX7, SMC2, SUMO1</i>	Human PMCs	CPO	(A. Suzuki, Li, et al., 2019)
miR-374a-5p	<i>ARNT, BMP2, CRISPLD1, FGFR2, JARID2, MSX1, NOG, RHPN2, RUNX2, WNT5A, ZNF236</i>	Human PMCs	CPO	(A. Suzuki, Li, et al., 2019)
miR-4680-3p	<i>ERBB2, JADE1, MTHFD1, WNT5A</i>		CPO	
miR-497-5p	<i>BAG4, CHD7, FGFR1, FOXP2, HECTD1, RUNX2, TFAP2A</i>	Human lip fibroblasts	CL/P	(Gajera et al., 2019)
miR-655-3p	<i>BCL2, CYP1A1, DMD, FZD6, HOXB3, MID1, NTN, SATB2</i>		CL/P	
miR-124-3p	<i>CDC42, IFT88, PBX3</i>	Mouse LMCs	CLO	(A. Suzuki, Yoshioka, et al., 2019)

miRNA	Target genes	Models	OFC subtype	References
	<i>BMPR1A</i> , <i>CDC42</i> , <i>IFT88</i> , <i>PBX3</i> , <i>TGFBR1</i> , <i>ZEB1</i>	Mouse O9–1 cells		
miR-106a-5p	<i>TGFBR2</i>	Mouse PMCs	CPO	(Zhang et al., 2020)

* miR-17–92 mouse mutants had incomplete penetrance of orofacial cleft. Compound miR-17–92/miR-106b-25 mutants had complete penetrance.

Table 4.

List of individual miRNAs associated with OFCs in human population or patient-derived tissue studies. CL/P, cleft lip with or without cleft palate; CLO, cleft lip only; CPO, cleft palate only; OFC, orofacial cleft.

miRNA	Target genes	Populations	Associated OFCs	References
miR-140	-	Han Chinese individuals OFC: n = 557 NSD: n = 306	CPO	(L. Li et al., 2010)
	<i>PDGFRA</i>	Han Chinese Individuals CPO: n = 169 NSD: n = 306	CPO	(L. Li et al., 2011)
	<i>PDGFRA</i>	Thai individuals CPO: n = 102 NSD: n = 500	CPO	(Rattanasopha et al., 2012)
miR-3649	<i>MSX1</i>	Individuals from China OFC: n = 602 CLO: n = 238 CPO: n = 56 CL/P: n = 305 NSD: n = 605	CL/P	(L. Ma et al., 2014)
miR-145	<i>FGF5</i>	Individuals from China OFC: n = 602 CLO: n = 232 CPO: n = 49 CL/P: n = 289 NSD: n = 605	CLO, CPO	(D. Li et al., 2016)
miR-187	<i>FGF9</i>		OFC	
miR-469	<i>FGF2</i>		CLO, CL/P	
miR-423-3p	<i>FOXE1</i>	Individuals from China OFC: n = 602 NSD: n = 605 No n values given for subtypes	CLO	(Yin et al., 2016)
miR-24-3p	-	Individuals from China CL/P: 4 NSD: 4	CL/P	(S. Wang et al., 2017)
miR-1260b	-			
miR-205-5p	-			
miR-18a-5p	-	Dutch children 29 months old or younger CL/P: n = 8 CPO: n = 5 NSD: n = 10	CPO	(Schoen et al., 2018)
miR-181a	-			
miR-29c-5p	-			
miR-3182	-			
miR-451a	-			
miR-549a	-			
miR-92a-3p	-			
miR-92b-5p	-			
miR-93-5p	-			
miR-505-3p	-		CL/P	
miR-146a	<i>TRAF6</i>	Individuals from China OFC: n = 1406 CL/P: n = 1197 CPO: n = 206 NSD: n = 1578	CL/P CPO	(Pan et al., 2018)