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Developmental MicroRNA Expression Profiling of Murine Embryonic Orofacial Tissue

Partha Mukhopadhyay, **Guy Brock**, **Vasyl Pihur**, **Cynthia Webb**, **M. Michele Pisano***, and **Robert M. Greene**
University of Louisville Birth Defects Center, Department of Molecular Cellular and Craniofacial Biology, ULSD, University of Louisville, Louisville, Kentucky

Abstract

BACKGROUND—Orofacial development is a multifaceted process involving precise, spatio-temporal expression of a panoply of genes. MicroRNAs (miRNAs), the largest family of noncoding RNAs involved in gene silencing, represent critical regulators of cell and tissue differentiation. MicroRNA gene expression profiling is an effective means of acquiring novel and valuable information regarding the expression and regulation of genes, under the control of miRNA, involved in mammalian orofacial development.

METHODS—To identify differentially expressed miRNAs during mammalian orofacial ontogenesis, miRNA expression profiles from gestation day (GD) -12, -13 and -14 murine orofacial tissue were compared utilizing miRXplore microarrays from Miltenyi Biotech. Quantitative real-time PCR was utilized for validation of gene expression changes. Cluster analysis of the microarray data was conducted with the clValid R package and the UPGMA clustering method. Functional relationships between selected miRNAs were investigated using Ingenuity Pathway Analysis.

RESULTS—Expression of over 26% of the 588 murine miRNA genes examined was detected in murine orofacial tissues from GD-12–GD-14. Among these expressed genes, several clusters were seen to be developmentally regulated. Differential expression of miRNAs within such clusters were shown to target genes encoding proteins involved in cell proliferation, cell adhesion, differentiation, apoptosis and epithelial-mesenchymal transformation, all processes critical for normal orofacial development.

CONCLUSIONS—Using miRNA microarray technology, unique gene expression signatures of hundreds of miRNAs in embryonic orofacial tissue were defined. Gene targeting and functional analysis revealed that the expression of numerous protein-encoding genes, crucial to normal orofacial ontogeny, may be regulated by specific miRNAs.

Keywords

orofacial; microRNA; microarray; fetal; development

*Correspondence to: M. Michele Pisano, Birth Defects Center, University of Louisville, ULSD, 501 S. Preston Street, Suite 301, Louisville, KY 40292., pisano@louisville.edu.

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INTRODUCTION

Orofacial clefting, which includes congenital structural abnormalities of the lip and/or palate, is one of the most prevalent birth defects with a frequency of 1 in 1000 live births for cleft lip (with or without cleft palate) and 1 in 2000 live births for isolated cleft palate (Schutte and Murray, 1999; Spritz, 2001). The facial region in mammalian embryos develops primarily from the frontonasal prominence, the nasal processes, and the first branchial arch. The bilateral maxillary processes of the first arch enlarge and fuse with the medial nasal processes, thereby forming the entire upper lip. The palatal processes develop from the oral aspect of each maxillary process, make contact, fuse with one another along their anterior-posterior length, and thereby give rise to the secondary palate or roof of the oral cavity. Normal morphogenesis of the orofacial region is reliant upon proper spatiotemporal patterns of mesenchymal cell proliferation, apoptosis, tissue differentiation, and the synthesis and degradation of extracellular matrix components (Greene and Pisano, 2004; Greene and Pisano, 2005). Furthermore, an array of molecular autocrine and paracrine interactions, in conjunction with epithelial-mesenchymal transformation, are also critical for orofacial ontogenesis (Richman and Tickle, 1989; Greene et al., 1998; Greene and Pisano, 2004; Greene and Pisano, 2005). Each of these events necessitates the expression of numerous genes whose precise regulation is central to normal orofacial development.

Numerous small RNAs (18–25 bases in length), termed microRNAs (miRNAs), have been found to execute key functions in silencing expression of specific target genes in both plant and animal systems (for review, see Wang et al., 2007; Zhang et al., 2007). MicroRNAs regulate expression of genes post-transcriptionally through binding and then directly inhibiting the translation, and/or resulting in the destabilization, of their target mRNAs (for review, see Pan and Chegini, 2008; Bartel, 2009). Experimental findings from numerous studies established crucial functions of miRNAs in regulating many developmental events such as proliferation, differentiation, and apoptosis during embryogenesis (for review, see O'Rourke et al., 2006; Yang and Wu, 2006). The role of miRNAs in craniofacial development is beginning to emerge. In zebrafish, microRNA 140 (Mirn140) was shown to inhibit platelet-derived growth factor receptor alpha-mediated attraction of cranial neural crest cells to the oral ectoderm (Eberhart et al., 2008), this process being essential for normal morphogenesis of the palate.

Our understanding of the mosaic of interacting signal transduction pathways associated with normal craniofacial development has expanded significantly in the last several years. Members of the transforming growth factor β family (TGFβs), bone morphogenetic proteins (BMPs), cAMP, epidermal growth factor (EGF), mitogen-activated protein kinases, Wnts, and retinoic acid, as well as a range of downstream transcriptional regulators such as Smads, CREB, CBP, p300, Ski, SnoN, NcoRs, and HDACs, have been shown to interact and participate in this dynamic process (Gehris and Greene, 1992; Mendelsohn et al., 1994; Brunet et al., 1995; Weston et al., 1998; Warner et al., 2002; Lan et al., 2006; Mukhopadhyay et al., 2006). An increasing number of miRNAs have been shown to target many of the aforementioned signaling mediators (Kawasaki and Taira, 2003; Li and Carthew, 2005; Martello et al., 2007; Kennell et al., 2008; Zhao et al., 2008; Lin et al., 2009) affecting diverse cellular processes. For instance, TGFβ-mediated epithelial-mesenchymal transformation (EMT), a process critical to normal development of the secondary palate (Sun et al., 1998), was shown to be associated with marked down-regulation of miR-205 and all five members of the micro-RNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) (Gregory et al., 2008). Importantly, induced expression of the miR-200 family alone was sufficient to *prevent* TGF-beta-induced EMT.

MicroRNA microarray technology has been successfully exploited to generate “microRNA gene expression profiles” of the cell cycle (Corney et al., 2007), cell differentiation (Zhan et al., 2007), cell death (Kren et al., 2009), embryonic development (Mineno et al., 2006; Hicks et al., 2008), stem cell differentiation (Lakshmipathy et al., 2007), different types of cancers (Gottardo et al., 2007; Wu et al., 2009), the diseased heart (Tatsuguchi et al., 2007), and normal as well as diseased neural tissue (Miska et al., 2004; Ferretti et al., 2009). Thus, microRNA gene expression profiling offers an effective means of acquiring novel and valuable information regarding the expression and regulation of genes, under the control of miRNA, involved in mammalian orofacial development.

METHODS

Animals

Mature male and female ICR mice (Harlan, Indianapolis, IN), maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility, on a 12-hour light/dark cycle and provided *ad libitum* food and water, were mated overnight. The presence of a vaginal plug the following morning was considered as evidence of mating, and the time designated as gestational day 0 (GD-0). On GD-12, GD-13, and GD-14, which represent the critical period of palate development in the mouse, female mice were euthanized by asphyxiation and embryos were dissected from uteri in sterile calcium/magnesium-free PBS. Extraembryonic membranes were removed from the embryos, and first branchial arch–derived tissue, including primary and secondary palatal tissue, was excised as shown in Figure 1 and as previously described (Gehris et al., 1991; Mukhopadhyay et al., 2006). Excised tissue was minced and stored at minus 80°C in PrepProtect Stabilization Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) for subsequent shipment to Miltenyi Biotec for miRNA expression analysis. For each day of gestation, three independent pools of 15 to 20 staged embryos were used to procure embryonic orofacial tissues for preparation of three distinct pools of RNA that were independently processed and applied to individual miRXplore micro-RNA Microarray chips (Miltenyi Biotec).

RNA Extraction and Microarray Hybridization

Total RNA (containing miRNAs) was isolated using standard RNA extraction protocols. The quality and quantity of total RNA samples were determined by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). The RNA Integrity Numbers (RINs) of all the RNA samples were between 9.7 and 10.0. RINs greater than 6 represent RNA of sufficient quality for miRNA microarray experiments (Fleige and Pfaffl, 2006). RNA samples (1 μg) isolated from mouse embryonic orofacial tissues (GD-12–GD-14) as well as the miRXplore Universal Reference (control) were fluorescently labeled with Hy5 (red) or Hy3 (green), respectively, and hybridized to miRXplore Microarrays (Miltenyi Biotec) using the a-Hyb Hybridization Station (Miltenyi Biotec). Probes for a total of 1336 mature miRNAs (from human, mouse, rat, and virus), including positive control and calibration spots, were spotted in quadruplicate on each microarray. Each array included probes for 588 murine miRNAs. The miRXplore Universal Reference controls, provided by Miltenyi, represent a defined pool of synthetic miRNAs for comparison of multiple samples. Fluorescence signals of the hybridized miRXplore Microarrays were detected using a laser scanner from Agilent Technologies.

Microarray Preprocessing

Mean and median signal and local background intensities for the Hy3 and Hy5 channels were obtained for each spot on each of the nine microarray images using the ImaGene software (BioDiscovery, El Segundo, CA). Low-quality spots were identified and given

relative weights, which were subsequently used in data analysis and modeling of miRNA expression values. A total of 5404 spots remained after discarding empty spots, dummy spots, extreme outliers, and those with missing background intensities. The pre-processing procedure consisted of three steps. First, all spots for which the mean foreground intensity was smaller than 1.1 times the mean background intensity in three or more arrays were discarded, unless all three samples came from the same day. Several within-array normalization procedures on the remaining 864 spots were evaluated. Based on visual inspection of the mean log2 expression values versus the log2 expression ratio for each spot ("MA" plots), it was decided that "no within-array" normalization gave the best results, and the 72 control spots were subsequently removed. In the second step, a simple background correction was performed by subtracting background from the foreground intensities. Log2 ratios of Hy5 (red, experimental) versus Hy3 (green, control) expression were calculated for each spot. Seven spots (in all nine arrays) exhibited negative intensities after the background procedure. These were treated as missing and then imputed using the K-nearest neighbor imputation scheme (Troyanskaya et al., 2001). Last, the between-array normalization using the quantile method was performed to align the distributions of the log-ratios on each chip (Bolstad et al., 2003; Rao et al., 2008). Visual inspection of heat maps and hierarchical clustering of the samples revealed that one replicate from each day did not cluster with the other replicates on that day. Because the other two replicates for each day were highly correlated ($r > 0.98$), we decided to use only those two replicates from each day for subsequent statistical analysis. Each spot is replicated on each chip 4 times, and so we maximized statistical power by using a hierarchical linear model that incorporated the replicate information on each chip. After all preprocessing steps, the total number of miRNA left was 198. The R package *limma* (Smyth, 2005) from the Bioconductor project (http://www.bioconductor.org) was used for all preprocessing steps, as well as all statistical analyses. The experimental data are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20880; platform accession no. GPL10179).

Statistical Analysis of miRNA Data

To identify miRNAs that are expressed by developing murine orofacial tissue on GD-12, GD-13, and GD-14, the four replicates on each chip and the three arrays corresponding to each of the three gestation days ($n = 12$) were averaged. Because all expression values were represented as log2 ratios of experimental versus universal reference, two criteria were used to determine miRNA expression. The first criterion was that the log-ratio was larger than zero, and the second was that the signal intensity in the experimental sample (red label) was in the 50th percentile or greater of its distribution. Differential expression of miRNAs between different days of gestation was determined by fit to a hierarchical linear model using the *limma* package (Smyth, 2004) and testing the corresponding contrasts of interest (e.g., GD-12 vs. GD-13, GD-13 vs. GD-14, and GD-12 vs. GD-14) for each miRNA. Each spot was weighted in a fashion inversely proportional to its quality score, and duplicate spots on each chip were taken into account using the duplicate correlation option (Smyth et al., 2005). Fold change, adjusted *t*-statistic, and unadjusted and FDR adjusted *p* values were calculated for each miRNA for each comparison. All miRNAs with adjusted *p* values below 0.05 were considered as differentially expressed.

After identifying the differentially expressed miRNAs, clustering analysis was performed to group genes with similar trends over time. The R package *clValid* (Brock et al., 2007) was used to evaluate several clustering algorithms (UPGMA [hierarchical clustering], SOM, SOTA, DIANA, and model-based clustering; Kaufman and Rousseeuw, 1990; Handl et al., 2005) using a number of validation measures. The clustering algorithm and number of

clusters that gave the best results on the validation measures were selected to visually display the data.

MicroRNA Target Analysis

MicroRNA target analysis was performed separately for all genes expressed on either one of the gestation days GD-12, GD-13, and GD-14, and for the miRNAs that were differentially expressed during murine orofacial ontogenesis (GD-12–GD-14). Only murine miRNAs (those with a MMU prefix) were selected for analysis. miRNA targets were mined using the miRDB (http://mirdb.org/miRDB/), an online database for miRNA target prediction in animals. The database is populated by the targets predicted by the computational tool MirTarget2 (Zhao et al., 2008). The miRNA target-scoring system utilized by the miRDB assigns scores to all three UTRs with seed-matching sites. If the number of candidate sites per single UTR is larger than one, then all these sites are combined and a single score is computed. The scores were computed using the following the equation:

$$S = 100\left(1 - \prod_{i=1}^{n} P_i\right).$$

where P_i represents the p values for each candidate site as estimated by the SVM, and n represents the number of candidate sites per single UTR. An ordered list of miRNA-gene symbol pairs sorted by their target scores were thus obtained. Target analysis of selected differentially expressed miRNAs (whose expression was verified by TaqMan QRTPCR; see Results) was achieved using the miRDB online database (http://mirdb.org/miRDB/). To discuss the likely physiological role(s) of specific differentially expressed miRNAs in orofacial development, gene targets of such miRNAs were also verified with three other miRNA target prediction software programs: PicTar (http://pictar.mdc-berlin.de), TargetScan (http://www.targetscan.org), and miRanda (http://www.microrna.org/microrna/home.do). Predicted targets of differentially expressed miRNAs were assessed for enrichment in GO categories and involvement in biological pathways using DAVID (Dennis et al., 2003; Huang et al., 2009) and Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA).

Quantitative Real-Time PCR (TaqMan)

Total RNA (containing miRNAs) from GD-12, GD-13, or GD-14 orofacial tissue was isolated using the miR-VANA microRNA isolation kit (Applied Biosystems–Ambion, Foster City, CA). cDNA was synthesized with miRNA-specific looped reverse transcription (RT) primer using the TaqMan MicroRNA RT kit (Applied Biosystems). Real-time PCR (TaqMan) analysis was performed on a TaqMan ABI Prism 7000 Sequence Detection System (Applied Biosystems). Matching primers and fluorescence probe for each miRNA were purchased from Applied Biosystems. For each miRNA analyzed, 900 nM of forward and reverse primers was utilized. The final concentration of the fluorescent probe was 200 nM. The PCR reaction was performed in a total volume of 25 µl containing 0.2 mM dATP, dCTP, and dGTP, 0.4 mM dUTP, 0.625 unit of Amplitaq Gold, and 2 µl of cDNA template. Cycling parameters were 95°C for 10 minutes for denaturation of DNA strands, followed by 40 cycles of denaturation at 95°C for 15 seconds each, and primer extension at 60°C for 1 minute. Data were acquired and processed with Sequence Detection System 1.0 software (Applied Biosystems). Each cDNA sample was tested in triplicate, and mean Ct values are reported. Primers and fluorescent probe for sno-rna-202, which was used as a housekeeping standard, were obtained from Applied Bio-systems. For purposes of quantification, each miRNA was normalized to the amount of sno-rna-202 PCR product present in each sample.

RESULTS

MicroRNAs derived from three independent sets of GD-12, GD-13, and GD-14 mouse embryonic orofacial tissue (nine total samples) and miRXplore Universal Reference (synthetic RNA pools used as controls) were fluorescently labeled with Hy5 (red) or Hy3 (green), respectively, and were used to probe separate miRXplore microarrays containing oligonucleotide probes representing a total of 1336 mature miRNAs (from human, mouse, rat, and virus), of which 588 were of murine origin. After preprocessing of the data, 68, 72, and 66 miRNAs were found to be expressed in the developing orofacial region on GD-12, GD-13, and GD-14, respectively (Table 1). Among the expressed miRNAs, 57 were common to all 3 days of gestation. Mir-674, -18B, and -301A were uniquely expressed on gestational day 12, mir-411 was uniquely expressed on GD-13, and mir-22 and let-7d were uniquely expressed on GD-14. Numerous miRNAs exhibited differential temporal expression patterns. Differentially expressed miRNAs with adjusted *p* values below 0.05 and with fold changes (in gene expression) are shown in Table 2 for GD-13 compared to GD-12, in Table 3 for GD-14 compared to GD-12, and in Table 4 for GD-14 compared to GD-13. Twelve miRNAs demonstrated enhanced and 14 miRNAs demonstrated reduced expression in GD-13 orofacial tissue when compared to GD-12. Similarly, 39 miRNAs demonstrated increased and 47 miRNAs demonstrated decreased expression in GD-14 orofacial tissue when compared to GD-12. Eighteen miRNAs demonstrated higher and 24 miRNAs demonstrated lower expression in GD-14 orofacial tissue when compared to GD-13. The complete list of fold changes, *t*-statistics, and *p* values associated with each miRNA passing our statistical filter (198 miRNAs in total) for each comparison of gestational days is presented in Supplementary Tables 1–3.

Utilization of the clValid R software package and applying the UPGMA (hierarchical) clustering algorithm to the data revealed distinct patterns of microRNA expression during murine orofacial ontogenesis. Using the validation measures found in the clValid R package, several recognizable temporal clusters of miRNAs were identified (Fig. 2). Six separate cluster profiles representing distinguishable patterns of miRNA expression were defined (Fig. 3). MicroRNAs comprising each cluster have been tabulated on Supplementary Table 4. Among the expressed miRNAs are members of the let-7, mir-15, -16, -18, -19, mir-20–27, -30, mir-92–93, -96, -99, 106, -125, -126, -130, -140, -141, -145, -148, -152, -181, -188, -193, -199, -200, -205, -214, -218, -301, -322, -335, -347, -376, -411, -424, -429, -451, -674, and -744 miRNAs families (Table 1). Target analysis of selected differentially expressed miRNAs (whose expression has been verified by TaqMan QRTPCR; see Table 5) using the miRDB online database (http://mirdb.org/miRDB/) revealed that miRNAs exhibiting developmentally regulated expression target a panoply of genes encoding molecular markers, such as cytoskeletal and extracellular matrix proteins, growth and differentiation factors, signal transduction modulators and effectors, and transcription factors (Supplementary Data File 1). Intriguingly, many of these target genes have been reported to be either associated with, or indispensable for, mammalian orofacial ontogenesis (Table 6). Furthermore, enriched gene ontology biological processes for the putative miRNA targetgenes, as predicted by the DAVID software (http://david.abcc.ncifcrf.gov/), revealed that several target genes are either documented or likely to be linked with cellular processes vital to morphogenesis of the embryonic orofacial tissue (Table 7). Finally, target analysis of miRNAs representing specific clusters, using the miRDB online database (http://mirdb.org/miRDB/), identified potential gene targets associated with vital stage-specific cellular events central to normal palatogenesis (Table 8).

MicroRNA gene expression profiling results were independently validated using TaqMan quantitative real-time PCR (Bustin, 2000). Expression levels of 13 candidate miRNAs, exhibiting diverse patterns of differential temporal regulation, were quantified and compared

to those levels determined by microarray. Expression profiles of 11 of the 13 miRNA genes tested were found to be in consistent agreement between the two methods (Table 5).

To determine how the differentially expressed genes encoding miRNAs and their target genes might interact in the developing murine orofacial tissue, an Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA) was performed using the Ingenuity Systems program (www.ingenuity.com). IPA employs a knowledge base program to generate relevant and interacting biological networks. One representative network with eight differentially expressed miRNAs (mir-20a, mir-20b, mir-22, mir-106a, mir-140, mir-206, and mir-342) is presented in Figure 4. Interestingly, this gene network also contains several direct and indirect target genes (of the differentially expressed miRNAs), known to be expressed in embryonic orofacial tissue and to execute crucial roles during orofacial ontogenesis (Fig. 4). Overlay of the network with relevant biological functions highlighted numerous cellular processes crucial for orofacial growth and maturation within the aforementioned miRNA-mRNA gene network (Fig. 5). Likewise, overlay of the network with canonical pathways led to the emergence of a range of signal transduction pathways (e.g., TGFβ-, BMP-, VEGF-, retinoic acid-, JAK/Stat-, and p53 signaling) operative within the gene network (Fig. 6). This reinforces the notion that crosstalk between diverse signal transduction pathways cooperatively orchestrates the morphogenesis of the embryonic orofacial tissue. Finally, to demonstrate that the differentially expressed miRNAs could be directly linked to potential target genes already reported to be associated with orofacial development, two more gene networks, one with miRNA genes exhibiting enhanced expression (Fig. 7) and the other displaying diminished expression (Fig. 8) in GD-13 versus Gd-12 orofacial tissue, were developed utilizing IPA and the miRDB online database (http://mirdb.org/miRDB/).

DISCUSSION

MicroRNAs (miRNAs) represent the largest family of noncoding RNAs involved in gene silencing (for review see Conrad et al., 2006; Lee et al., 2006; Dykxhoorn, 2007; Voinnet, 2009). Expression of approximately one-third of all human genes is thought to be regulated by miRNAs. Members of this RNA family are critical regulators of cell and tissue differentiation during embryogenesis (for review see Conrad et al., 2006; Lee et al., 2006; Mineno et al., 2006). For example, inactivation of C. elegans Dicer (dcr-1), an RNase III enzyme that cleaves double-stranded RNA or miRNA precursors into mature siRNAs or miRNAs (Lee et al., 2002), results in heterochronic phenotypes (Grishok et al., 2001). Knocking down the gene encoding Dicer in zebrafish results in overall growth arrest during embryogenesis (Wienholds et al., 2003). This is perhaps not surprising because the Drosophila Dicer-1 (DCR-1) is required for G1/S transition (Hatfield et al., 2005). Indeed, loss of murine DCR-1 results in embryolethality (Bernstein et al., 2003; Yang et al., 2005).

Except for two reports (Lu et al., 2005; Eberhart et al., 2008), the role of miRNAs in craniofacial development has been almost entirely unexplored. Understanding miRNA function, particularly during embryogenesis, initially requires a detailed determination of expression patterns. This is particularly important because expression of avian (Darnell et al., 2006; Xu et al., 2006), zebrafish (Giraldez et al., 2005), and murine (Takada et al., 2006) miRNAs is tightly controlled in a tissue- and developmental stage-specific manner during organogenesis (Plasterk, 2006; Saetrom et al., 2007). Thus, the expression profiles of 1336 mature miRNAs were examined in developing orofacial tissue. Expression profiles of 154 miRNAs, of which 90 were distributed in specific temporal patterns, are reported here.

Morphogenesis of the mammalian orofacial region necessitates coordination of several key physiological processes (such as cellular proliferation, differentiation, and apoptosis, among

others) governed by numerous genes encoding a panoply of growth factors/signaling mediators, transcriptional regulators, and extracellular matrix proteins (Richman and Tickle, 1989; Greene et al., 1998; Greene and Pisano, 2004; Greene and Pisano, 2005). Recent scientific evidence clearly suggests that an array of miRNAs potentially regulate each of these events by targeting the genes responsible in modulating these cellular processes. To ensure that those genes, targeted by various differentially expressed miRNAs in the developing orofacial tissue, could be linked to the aforementioned critical cellular events, gene ontology (GO) enrichment analysis was performed exploiting the GO database (http://www.geneontology.org/) and the DAVID software (http://david.abcc.ncifcrf.gov/). It is evident from the results presented in Table 7 that numerous predicted (many of which are also experimentally verified) target genes of selected, developmentally regulated miRNAs are indeed (or likely to be) associated with key cellular events such as cell proliferation, migration, differentiation, apoptosis, synthesis of extracellular matrix molecules, and epithelial-mesenchymal transformation, which are all indispensable for mammalian orofacial ontogenesis.

A balance between the processes of cell proliferation, apoptosis, and terminal cell differentiation is crucial for ensuring normal development of the orofacial region. A number of genes encoding apoptosis-associated proteins, such as peptidoglycan recognition protein, TNF3-like protein, neuronal death protein Bid-3, TNF-related apoptosis inducing ligand (TRAIL), TNF receptor superfamily member 5 or CD40, caspase-9S, -12, -14, and an activator of caspases known as DIABLO are expressed in orofacial tissue during its development (Mukhopadhyay et al., 2004). MicroRNAs mir-193 and let-7c have been reported to affect the activation of the caspase cascade and subsequent apoptosis in human cancer cells (Ovcharenko et al., 2007). Mir-193 has also been predicted to target bone morphogenetic protein receptor, type 1A (BMPR1a), a BMP signaling mediator indispensable for orofacial development as *Bmpr1a* mutant embryos present with cleft lip with palate and exhibit arrested tooth formation (Liu et al., 2005). Another likely target of mir-193 (according to the miRDB online database) is the gene encoding Engrailed 2, which has been implicated in craniofacial and neural tube development (Augustine et al., 1995; Knight et al., 2008). In the current study, mir-193 and another closely related miRNA, mir-193b, demonstrated significantly elevated expression in developing orofacial tissue (GD-12–GD-14), suggesting that these miRNAs regulate specific downstream target genes during orofacial ontogenesis (Tables 2–5).

The let-7 family of miRNAs is well conserved across species and their sequences, and functions are highly conserved in mammals (Bussing et al., 2008; Roush and Slack, 2008). Diverse biological functions for the let-7 family of miRNAs have been described previously. These include cellular proliferation and differentiation (Yu et al., 2007; Bussing et al., 2008; Roush and Slack, 2008; Sokol et al., 2008), apoptosis (Ovcharenko et al., 2007), regulation of stem cell differentiation (Reinhart et al., 2000), limb morphogenesis (Lancman et al., 2005), and neuromusculature development (Caygill and Johnston, 2008; Sokol et al., 2008). In addition to its role as an apoptotic inducer, let7c has also been reported to function as an antiproliferative mediator by controlling levels of the c-myc oncogene (Yang et al., 2008). Several likely target genes of the let-7 family of miRNAs, encode proteins (E2F transcription factor 5, neuroblastoma ras oncogene, growth differentiation factor 6, and cyclin J) known to be involved in cell proliferation. Additional probable let-7 target genes encode growth factors/factor receptors as well as transcription factors (ALK5, ALK7, TGFβ receptor III, activin A receptor, IGF1R, IGF2BP2, IGF2BP3, Hand-1) known to be either associated with, or crucial for, orofacial morphogenesis (Ferguson et al., 1992; Melnick et al., 1998; Taya et al., 1999; Dudas et al., 2006; Barbosa et al., 2007; Pravtcheva and Wise, 2008). In the current study, genes encoding four members of the let-7 family of miRNAs (let-7b, -7c, -7d, and -7i) demonstrated significant differential regulation, during orofacial

development (Tables 1–4), suggesting a key role for these miRNAs during orofacial ontogeny.

Ligands of the platelet-derived growth factor (Pdgf) family and their receptors are crucial regulators of embryogenesis. Platelet-derived growth factor receptor alpha (Pdgfra) signaling is integral for tooth cusp and palate morphogenesis regulating epithelial-mesenchymal interaction during tooth and palate morphogenesis (Xu et al., 2005). Interestingly, Pdgf-mediated attraction of cranial neural crest cells to palatal oral ectoderm has been reported to be under control of mir-140 (Eberhart et al., 2008). Consistent with this observation is our observation that the gene encoding mir-140 demonstrated significantly increased expression in developing orofacial tissue (GD-12–GD-14) precisely during the period of palatal development (Tables 2–5). In addition, three miRNA target prediction software programs (PicTar, Target Scan, and miRanda) predicted several other genes (Jagged-1 [Jag-1], Sox-4, and GDF-6), reported as important contributors to orofacial development (Pilia et al., 1999; Maschhoff et al., 2003; Juriloff and Harris, 2008; Chiquet et al., 2009), as targets of mir-140. Taken together, these observations suggest an important role for mir-140 during orofacial ontogenesis.

The steroid hormone 17β-estradiol (E2) is thought to regulate a number of developmental processes (Bookout et al., 2006; Heldring et al., 2007). Hormonal functions are generally mediated by two nuclear receptors, estrogen receptor α (ERα or Esr1) and ERβ (Esr2), whose levels are regulated developmentally and in a tissue-specific manner (Heldring et al., 2007). Extant evidence supports the premise that ESR1 plays a significant role in orofacial development (Matsuda et al., 2001; Greene et al., 2003; Ferrer et al., 2005; Osoegawa et al., 2008). Two miRNA target prediction software programs, PicTar and TargetScan, predicted Estrogen receptor (Esr-1) as one of the several targets of mir-22. A recent study (Pandey and Picard, 2009) reported that miR-22 strongly represses ER alpha expression by directly targeting the ER alpha mRNA 3′ UTR.

Fibroblast growth factors (FGFs) represent a large family of paracrine and autocrine proteins that function in controlling cell differentiation, proliferation, survival, and motility (Basilico and Moscatelli, 1992). Six members of this family (FGF-1, -2, -4, -5, -8, and -12) have been localized in, and thought to regulate outgrowth of, the developing facial primordia. In humans, mutations in FGF receptor genes (e.g., *Fgfr2*) have been linked to disorders with craniofacial anomalies such as Crouzon, Pfeiffer, and Apert syndromes as well as craniosynostosis (Francis-West et al., 1998; Riley et al., 2007). Moreover, several FGFR2 missense mutations were identified in subjects with nonsyndromic cleft lip and palate (Rice et al., 2004). Palatal clefting, due to failure of the palatal processes to grow, has been reported in homozygous $Fgfr2b^{-/-}$ knockout mouse embryos (Osoegawa et al., 2008). Recent evidence suggests that FGF signaling via FGFR2 in the oral epithelium is essential for cellular proliferation during tooth and palate development (Hosokawa et al., 2009). PicTar, TargetScan, and miRanda predicted Fgfr2 as one of the several targets of mir-22.

These programs also predicted Max (Myc-associated factor) as another target of mir-22. A protein functionally related to Max (Max-interacting protein or Mnt), a Mad-family bHLH transcription factor is deleted in Miller-Dieker syndrome (MDS), which presents with craniofacial dysmorphic features (Hirotsune et al., 1997). Loss of *Mnt* in mice also results in phenotypic outcomes similar to those observed in patients with MDS (Toyo-oka et al., 2004). These results support a key role for Mnt during embryogenesis, especially during craniofacial development. In the current study, the gene encoding mir-22 exhibited significantly enhanced expression in developing orofacial tissue (Tables 2–5). In view of the aforementioned findings, it can be argued that mir-22 executes key role(s) during orofacial development modulating expressions of its target genes encoding proteins operative in

processes such as estrogen signaling (Esr-1), FGF signaling (Fgfr2), and in the Myc/Max/Mad network (Max).

Recent studies suggest the importance of miRNAs, particularly mir-152, during craniofacial development. Fetal brains exhibited an increase in mir-152 expression following conditions of prenatal ethanol exposure, which suggests involvement in the pathogenesis of fetal alcohol syndrome (Wang et al., 2009). In the current study, a consistent and significant increase in expression of the gene encoding mir-152 was seen (Tables 2–5). The miRNA target prediction software programs, PicTar, TargetScan, and miRanda, predicted a number of genes (activin A receptor, type I [also known as Alk-2 or BMP type I receptor], Noggin, Inhibin-βB, Meox2, Mnt, and p300), thought to execute key roles in orofacial development, as potential targets of mir-152.

Bone morphogenetic proteins (BMPs) are critical in regulating maturation of embryonic orofacial tissue (Francis-West et al., 1994). Mice lacking *Alk2*, a target of mir-152 and the gene encoding one of the type I BMP receptors, exhibit multiple craniofacial defects including cleft palate (Dudas et al., 2004). BMP and noggin, a BMP antagonist, may also act in concert to regulate mesenchymal cell proliferation and their survival in the epithelium during frontonasal growth (Ashique et al., 2002). Moreover, the amount of noggin mRNA in the frontonasal prominences of mice with cleft lip (CL(+)) is significantly higher in comparison to that in mice without cleft lip (CL(−)) (Nakazawa et al., 2008). Other members of the TGFβ superfamily, such as activins, inhibins, and their related protein follistatin, are also known mediators of orofacial development, as mice with targeted deletions of these genes demonstrate various orofacial defects (Lambert-Messerlian et al., 2004). In addition, mice lacking both activin-beta A and activin-beta B (a target of mir-152) also display craniofacial defects (Matzuk et al., 1995). Additional evidence implicating mir-152 in regulation of craniofacial development includes the observation that the homeobox gene Meox-2, a target of mir-152, plays a role in palatal clefting (Jin and Ding, 2006). Collectively, these data support the notion that mir-152, modulating the expression of numerous target genes, including several members of the TGFβ superfamily, plays a defining role in outgrowth of the facial prominences and development of the orofacial region.

In the current study, the gene encoding mir-206 exhibited statistically significant and consistently elevated expression during orofacial development (Tables 2–5). Mir-206 has been reported to modulate the expression of *Utrn*, *Fstl1*, *Connexin 43*, and *Esr-1*, all reportedly involved in various aspects of craniofacial growth and maturation. *Utrn* encodes for utrophin, a large cytoskeletal protein thought to link F-actin to a transmembrane protein complex. Expression of *Utrn* in the embryonic neural tube and in neural crest-derived tissues such as facial ganglia and ossifying facial cartilages (Schofield et al., 1993) allows the suggestion of a role in orofacial development. Follistatin-like 1 (Fstl1), a distantly related homolog of the Activin and BMP antagonist Follistatin, has been identified as a regulator of early mesoderm patterning, somitogenesis, myogenesis, and neural development (Patel et al., 1996). We have previously reported expression of genes encoding follistatin-like 1 and follistatin-like 3, in developing murine orofacial tissue (Mukhopadhyay et al., 2006). The gap junction protein alpha 1 connexin (Cx43 or Connexin 43) participates in gap junctional communication essential for embryogenesis and patterning. Expressed in spatially restricted patterns in the developing facial primordia of the chick embryo (Minkoff et al., 1997), antisense oligo-based “knockdown” of Cx43 in the chick embryo has been reported to cause a range of developmental defects, including those in the craniofacial region (Becker et al., 1999).

Interestingly, target analysis of cluster 4 and cluster 6 miRNAs, displaying diminished expression within the developing orofacial tissue from GD-12 to GD-13 (Fig. 3), revealed several *potential* target genes involved in promotion of cell proliferation and/or mediation of signaling by crucial signaling mediators/growth factors (such as TGFβ, BMP, and Shh) (Table 8) that are indispensible for the initial phase of palatogenesis (when the bilateral palatal shelves grow and, hence, require growth factor signaling and gene expression) inducing, among other events, active cell proliferation (Richman and Tickle, 1989; Greene et al., 1998; Greene and Pisano, 2004; Greene and Pisano, 2005). These observations support the notion that miRNAs that target genes and growth factors involved in promotion of cellular proliferation are displaying decreased expression during this early phase of palate development (GD-12–GD-13), allowing their proliferation-inducing target genes to be expressed. Similarly, target analysis of cluster 5 miRNAs that exhibited reduced expression in embryonic orofacial tissue from GD-13 to GD-14 (Fig. 3) unveiled numerous, *prospective* target genes engaged in promotion of cellular events such as selective apoptosis, cellular adhesion, extracellular matrix reorganization, and cellular differentiation (Table 8) that are essential for the final phase of palate development when the bilateral palatal shelves proceed toward fusion and subsequently, following completion of fusion, initiate differentiation (e.g., chondrogenesis and osteogenesis) (Richman and Tickle, 1989; Greene et al., 1998; Greene and Pisano, 2004; Greene and Pisano, 2005). Thus, these findings underscore crucial developmental, stage-specific expression and potential cellular functions of discrete miRNAs during orofacial development including palatogenesis.

To pursue cause-consequence relationships between developmentally regulated miRNAs genes and genes targeted by these miRNAs, network/pathway reconstruction from our miRNA microarray data was performed utilizing Ingenuity Pathway Analysis (IPA) to assign these genes to specific cellular pathways. The program identifies biological relationships among genes/proteins of interest. One such gene network, involving eight differentially expressed miRNAs (mir-20a, mir-20b, mir-22, mir-206, mir-362, mir-106a, mir-152, and mir-140) whose expression was examined by both microarray and Taq-Man QRTPCR, is presented in Figure 4. This network is noteworthy for revealing biological processes such as cellular proliferation, growth of epithelial cells, and cell survival, known to be essential for orofacial development (Fig. 5). 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 (Fig. 6). Examples of such relevant pathways include the TGFβ-, BMP-, Wnt-, retinoic acid-, JAK/Stat-, VEGF-, PI3K/AKT-, aryl hydrocarbon receptor-, estrogen receptor, NFκβ-, and calcium-signal transduction pathways (Fig. 6). Additionally, several other signaling pathways within this network, regulating cell death (apoptosis-, p53-, and death receptor signaling), as well as cell survival and DNA damage (Nrf2-mediated oxidative stress response, hypoxia inducible factor 1α-, G1/S checkpoint regulation, G2/M DNA damage checkpoint regulation, etc.), were identified. Such pathway analysis emphasizes the significance of these miRNAs in governing cellular signal transduction events that direct normal growth and maturation of the embryonic orofacial region (Fig. 6).

Transcriptional repressors Cux1 and Cux2 belong to the family of CDP/Cut homeodomain factors that are widely expressed in mouse embryos and exhibit dynamic expression in craniofacial primordia during embryogenesis (Iulianella et al., 2003). Cux2 is thought to function as a transcriptional regulator inhibiting terminal differentiation and cell cycle exit, whereas Cux1 accelerates entry into the S phase of the cell cycle (Iulianella et al., 2003; Sansregret et al., 2006). Based on the gene network shown in Figure 6, *Cux1* is a common target of three miRNAs (mir-22, mir-206, and mir-140). This observation suggests a central regulatory role of these miRNAs in governing cell cycle progression during orofacial ontogenesis via modulation of *Cux1* expression.

IgLONs are GPI-anchored cell adhesion glycoproteins belonging to the immunoglobulin superfamily (McNamee et al., 2002). In the gene network represented in Figure 4 a new member of the IgLON family, *IgLON5*, is present as a common target of two differentially regulated miRNAs: mir-20a and mir-206. This implies crosstalk between this gene and the miRNAs that might play a yet unidentified role in orofacial ontogenesis. Support for this notion comes from the observation that IgLON family members are expressed in neural crest cells (Kimura et al., 2001).

A number of human morphogenetic disorders, several with craniofacial anomalies (e.g., Smith-Lemli-Opitz syndrome, desmosterolosis, X-linked chondrodysplasia punctata, CHILD syndrome), have been reported to be caused by abnormal cholesterol biosynthesis (Andersson, 2002). Children born with the Smith-Lemli-Opitz syndrome, a common autosomal recessive disorder, display specific facial dysmorphism and a range of congenital anomalies including palatal clefting and have a metabolic defect at the final step of the cholesterol biosynthetic pathway (Elias and Irons, 1995). Interestingly, an E3 ubiquitin ligase known as Mylip or Idol (inducible degrader of the LDLR), which has been implicated in cholesterol uptake, was a common target of several differentially expressed miRNAs (e.g., mir-20a, -20b, -106a, and -206) in our miRNA-mRNA gene network (Fig. 4). This observation points to possible regulation of cholesterol metabolism in the developing orofacial tissue by four developmentally regulated miRNAs via modulation of expression of one of their common target genes (*Mylip*).

Forkhead box transcription factors are three winged helix/fork-head DNA binding domain-containing transcriptional regulators. They comprise a large family of developmentally regulated transcription factors that contribute to embryonic pattern formation and regulation of tissue-specific gene expression (El-Hodiri et al., 2001). We have previously reported differentially regulated expression of the *foxc2, foxc1*, and *ches1* forkhead/winged helix transcription factor genes during orofacial ontogeny, suggesting that members of the forkhead box gene family are potential contributors to orofacial patterning (Mukhopadhyay et al., 2004). Indeed, mice with null mutations in *Foxc1* exhibit severe craniofacial dysmorphogenesis (Sommer et al., 2006), and mutations in the human *Foxc1* gene have been linked to a range of human syndromes with orofacial anomalies (e.g., Axenfeld-Rieger syndrome, syndromes with Peters Anomaly, Ritscher-Schinzel syndrome, and 6p25 deletion syndrome) (Maclean et al., 2005). Figure 4, which represents a miRNA-mRNA gene association map, reveals the emergence of *Foxc1* as an important target gene of several miRNAs differentially expressed in embryonic orofacial tissue.

The importance of the two CREB-binding proteins, CBP and p300, in orofacial growth and maturation has been discussed. Our miRNA-mRNA gene association map (Fig. 4) reveals CBP as a direct target of mir-362. Analysis with the miRNA target prediction software programs, PicTar, TargetScan, and miRanda, indicated that this differentially regulated miRNA (Tables 2–5) also targets Satb2 (special AT-rich sequence binding protein 2) and Osr2 (odd-skipped related 2), which have been directly linked to human syndromes as well as knockout mouse models with cleft palate and other orofacial defects (Lan et al., 2004; Van Buggenhout et al., 2005). Thus, mir-362 may represent a key modulator of orofacial maturation.

We have previously emphasized the importance of apoptosis in tissue morphogenesis during normal orofacial ontogeny by demonstrating the differential expression, in developing orofacial tissue, of numerous genes encoding apoptosis-associated proteins (TNF3-like protein, neuronal death protein Bid-3, TNF-related apoptosis inducing ligand [TRAIL], TNF receptor superfamily member 5 or CD40, caspase-9S, -12, -14, and an activator of caspases known as DIABLO) (Mukhopadhyay et al., 2004). Further clarification of the means by

which apoptosis may be regulated during orofacial development comes from the observation that Bcl2, an anti-apoptotic protein, appeared as a major node in the miRNA-mRNA gene association network and a direct target of mir-140, and an indirect target of several other developmentally regulated miRNAs (Fig. 3). These interactive gene network-based observations underscore the fact that miRNAs play a key role in regulating apoptosis in the developing orofacial region.

Finally, the importance of the miRNAs, differentially expressed within the developing orofacial region, as demonstrated in the current study, in governing expression of key genes indispensable for orofacial morphogenesis is convincingly highlighted by the two gene networks presented in Figures 7 and 8. These figures portray networks generated with the miRNAs displaying enhanced (Fig. 7) or diminished (Fig. 8) expression in the GD-13 versus GD-12 orofacial tissue, and their potential target genes, almost all of which have been previously reported to be crucial for development of the orofacial region. Many of these target genes have been linked to syndromes involving orofacial anomalies such as cleft lip with or without cleft palate. These two gene networks clearly document the paramount significance of the various developmentally regulated miRNAs in directing expression of key genes orchestrating orofacial ontogenesis.

It is clear that miRNAs are critical regulators of cell and tissue differentiation. Findings from this gene expression profiling study, where miRNAs are shown to exhibit distinct temporal expression patterns during orofacial development, strongly support the notion that specific miRNAs function as crucial regulators of gene expression at a critical period of orofacial ontogenesis. The results presented suggest the possibility that various miRNAs, differentially expressed during development of the embryonic orofacial region, regulate expression of numerous gene targets. Because individual miRNAs may target multiple mRNAs, complex, cooperatively interacting regulatory combinations are likely to exist.

CONCLUSIONS

Results from this study identify numerous investigational candidate miRNAs as potential target mRNAs involved in regulating key cellular processes governing orofacial ontogenesis. The systems biology approach employed in the present study has allowed identification of cellular response pathways, as well as biological themes, that underlie orofacial maturation. Collectively, the data support the notion that differentially expressed miRNAs, regulating crosstalk among diverse signal transduction pathways, govern tissue differentiation and morphogenesis of developing orofacial tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Andersson HC. Disorders of post-squalene cholesterol biosynthesis leading to human dysmorphogenesis. Cell Mol Biol (Noisy-le-grand). 2002; 48(2):173–177. [PubMed: 11990452]

Ashique AM, Fu K, Richman JM, et al. Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. Development. 2002; 129(19):4647–4660. [PubMed: 12223420]

Augustine KA, Liu ET, Sadler TW, et al. Antisense inhibition of engrailed genes in mouse embryos reveals roles for these genes in craniofacial and neural tube development. Teratology. 1995; 51(5): 300–310. [PubMed: 7482351]

Barbosa AC, Funato N, Chapman S, et al. Hand transcription factors cooperatively regulate development of the distal midline mesenchyme. Dev Biol. 2007; 310(1):154–168. [PubMed: 17764670]

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136(2):215–233. [PubMed: 19167326]

Basilico C, Moscatelli D. The FGF family of growth factors and oncogenes. Adv Cancer Res. 1992; 59:115–165. [PubMed: 1381547]

Becker DL, McGonnell I, Makarenkova HP, et al. Roles for alpha 1 connexin in morphogenesis of chick embryos revealed using a novel antisense approach. Dev Genet. 1999; 24(1–2):33–42. [PubMed: 10079509]

Bernstein E, Kim SY, Carmell MA, et al. Dicer is essential for mouse development. Nat Genet. 2003; 35(3):215–217. [PubMed: 14528307]

Bolstad BM, Irizarry RA, Astrand M, et al. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19(2):185–193. [PubMed: 12538238]

Bookout AL, Jeong Y, Downes M, et al. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell. 2006; 126(4):789–799. [PubMed: 16923397]

Brock GN, Pihur V, Datta S, et al. clValid, an R package for cluster validation. J Statistical Software. 2007; 25:4.

Brunet CL, Sharpe PM, Ferguson MW, et al. Inhibition of TGF-beta 3 (but not TGF-beta 1 or TGF-beta 2) activity prevents normal mouse embryonic palate fusion. Int J Dev Biol. 1995; 39(2):345–355. [PubMed: 7669547]

Bussing I, Slack FJ, Grosshans H, et al. let-7 microRNAs in development, stem cells and cancer. Trends Mol Med. 2008; 14(9):400–409. [PubMed: 18674967]

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2000; 25(2):169–193. [PubMed: 11013345]

Caygill EE, Johnston LA. Temporal regulation of metamorphic processes in Drosophila by the let-7 and miR-125 heterochronic micro-RNAs. Curr Biol. 2008; 18(13):943–950. [PubMed: 18571409]

Chiquet BT, Hashmi SS, Henry R, et al. Genomic screening identifies novel linkages and provides further evidence for a role of MYH9 in nonsyndromic cleft lip and palate. Eur J Hum Genet. 2009; 17(2):195–204. [PubMed: 18716610]

Conrad R, Barrier M, Ford LP, et al. Role of miRNA and miRNA processing factors in development and disease. Birth Defects Res C Embryo Today. 2006; 78(2):107–117. [PubMed: 16847880]

Corney DC, Flesken-Nikitin A, Godwin AK, et al. MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. Cancer Res. 2007; 67(18):8433–8438. [PubMed: 17823410]

Darnell DK, Kaur S, Stanislaw S, et al. MicroRNA expression during chick embryo development. Dev Dyn. 2006; 235(11):3156–3165. [PubMed: 17013880]

Dennis G Jr, Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003; 4(5):3.

Dudas M, Kim J, Li WY, et al. Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. Dev Biol. 2006; 296(2):298–314. [PubMed: 16806156]

Dudas M, Sridurongrit S, Nagy A, et al. Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. Mech Dev. 2004; 121(2):173–182. [PubMed: 15037318]

Dykxhoorn DM. MicroRNAs in viral replication and pathogenesis. DNA Cell Biol. 2007; 26(4):239–249. [PubMed: 17465890]

Eberhart JK, He X, Swartz ME, et al. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. Nat Genet. 2008; 40(3):290–298. [PubMed: 18264099]

El-Hodiri H, Bhatia-Dey N, Kenyon K, et al. Fox (forkhead) genes are involved in the dorso-ventral patterning of the Xenopus mesoderm. Int J Dev Biol. 2001; 45(1):265–271. [PubMed: 11291856]

Elias ER, Irons M. Abnormal cholesterol metabolism in Smith-Lemli-Opitz syndrome. Curr Opin Pediatr. 1995; 7(6):710–714. [PubMed: 8776024]

Ferguson MW, Sharpe PM, Thomas BL, et al. Differential expression of insulin-like growth factors I and II (IGF I and II), mRNA, peptide and binding protein 1 during mouse palate development: comparison with TGF beta peptide distribution. J Anat. 1992; 181(Pt 2):219–238. [PubMed: 1284245]

Ferrer VL, Maeda T, Kawano Y, et al. Characteristic distribution of immunoreaction for estrogen receptor alpha in rat ameloblasts. Anat Rec A Discov Mol Cell Evol Biol. 2005; 284(2):529–536. [PubMed: 15803481]

Ferretti E, De Smaele E, Po A, et al. MicroRNA profiling in human medulloblastoma. Int J Cancer. 2009; 124(3):568–577. [PubMed: 18973228]

Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Asp Med. 2006; 27(2–3):126–139.

Francis-West P, Ladher R, Barlow A, et al. Signalling interactions during facial development. Mech Dev. 1998; 75(1–2):3–28. [PubMed: 9739099]

Francis-West PH, Tatla T, Brickell PM, et al. Expression patterns of the bone morphogenetic protein genes Bmp-4 and Bmp-2 in the developing chick face suggest a role in outgrowth of the primordia. Dev Dyn. 1994; 201(2):168–178. [PubMed: 7873788]

Gehris AL, D'Angelo M, Greene RM, et al. Immunodetection of the transforming growth factors beta 1 and beta 2 in the developing murine palate. Int J Dev Biol. 1991; 35(1):17–24. [PubMed: 1714290]

Gehris AL, Greene RM. Regulation of murine embryonic epithelial cell differentiation by transforming growth factors beta. Differentiation. 1992; 49(3):167–173. [PubMed: 1618373]

Giraldez AJ, Cinalli RM, Glasner ME, et al. MicroRNAs regulate brain morphogenesis in zebrafish. Science. 2005; 308(5723):833–838. [PubMed: 15774722]

Gottardo F, Liu CG, Ferracin M, et al. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol. 2007; 25(5):387–392. [PubMed: 17826655]

Greene RM, Nugent P, Mukhopadhyay P, et al. Intracellular dynamics of Smad-mediated TGFbeta signaling. J Cell Physiol. 2003; 197(2):261–271. [PubMed: 14502566]

Greene RM, Pisano MM. Perspectives on growth factors and orofacial development. Curr Pharm Des. 2004; 10(22):2701–2717. [PubMed: 15320737]

Greene RM, Pisano MM. Recent advances in understanding transforming growth factor beta regulation of orofacial development. Hum Exp Toxicol. 2005; 24(1):1–12. [PubMed: 15727050]

Greene, RM.; Weston, W.; Nugent, P. Signal transduction pathways as targets for induced embryotoxicity. In: Slikker, W.; Chang, L., editors. Handbook of developmental neurotoxicology. San Diego: Academic Press; 1998. p. 119-139.

Gregory PA, Bert AG, Paterson EL, et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol. 2008; 10(5):593–601. [PubMed: 18376396]

Grishok A, Pasquinelli AE, Conte D, et al. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell. 2001; 106(1):23–34. [PubMed: 11461699]

Handl J, Knowles J, Kell DB, et al. Computational cluster validation in post-genomic data analysis. Bioinformatics. 2005; 21(15):3201–3212. [PubMed: 15914541]

Hatfield SD, Shcherbata HR, Fischer KA, et al. Stem cell division is regulated by the microRNA pathway. Nature. 2005; 435(7044):974–978. [PubMed: 15944714]

Heldring N, Pike A, Andersson S, et al. Estrogen receptors: how do they signal and what are their targets. Physiol Rev. 2007; 87(3):905–931. [PubMed: 17615392]

Hicks JA, Tembhurne P, Liu HC, et al. MicroRNA expression in chicken embryos. Poult Sci. 2008; 87(11):2335–2343. [PubMed: 18931185]

Hirotsune S, Pack SD, Chong SS, et al. Genomic organization of the murine Miller-Dieker/lissencephaly region: conservation of linkage with the human region. Genome Res. 1997; 7(6): 625–634. [PubMed: 9199935]

Hosokawa R, Deng X, Takamori K, et al. Epithelial-specific requirement of FGFR2 signaling during tooth and palate development. J Exp Zool B Mol Dev Evol. 2009; 312B(4):343–350. [PubMed: 19235875]

Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4(1):44–57. [PubMed: 19131956]

Iulianella A, Vanden Heuvel G, Trainor P, et al. Dynamic expression of murine Cux2 in craniofacial, limb, urogenital and neuronal primordia. Gene Expr Patterns. 2003; 3(5):571–577. [PubMed: 12971989]

Jin JZ, Ding J. Analysis of Meox-2 mutant mice reveals a novel post-fusion-based cleft palate. Dev Dyn. 2006; 235(2):539–546. [PubMed: 16284941]

Juriloff DM, Harris MJ. Mouse genetic models of cleft lip with or without cleft palate. Birth Defects Res A Clin Mol Teratol. 2008; 82(2):63–77. [PubMed: 18181213]

Kaufman, L.; Rousseeuw, PJ. Finding groups in data: an introduction to cluster analysis. New York: Wiley; 1990.

Kawasaki H, Taira K. Functional analysis of microRNAs during the retinoic acid-induced neuronal differentiation of human NT2 cells. Nucleic Acids Res Suppl. 2003; (3):243–244. [PubMed: 14510471]

Kennell JA, Gerin I, MacDougald OA, et al. The microRNA miR-8 is a conserved negative regulator of Wnt signaling. Proc Natl Acad Sci USA. 2008; 105(40):15417–15422. [PubMed: 18824696]

Kimura Y, Katoh A, Kaneko T, et al. Two members of the IgLON family are expressed in a restricted region of the developing chick brain and neural crest. Dev Growth Differ. 2001; 43(3):257–263. [PubMed: 11422291]

Knight RD, Mebus K, Roehl HH, et al. Mandibular arch muscle identity is regulated by a conserved molecular process during vertebrate development. J Exp Zool B Mol Dev Evol. 2008; 310(4):355–369. [PubMed: 18338789]

Kren BT, Wong PY, Sarver A, et al. MicroRNAs identified in highly purified liver-derived mitochondria may play a role in apoptosis. RNA Biol. 2009; 6(1):65–72. [PubMed: 19106625]

Lakshmipathy U, Love B, Goff LA, et al. MicroRNA expression pattern of undifferentiated and differentiated human embryonic stem cells. Stem Cells Dev. 2007; 16(6):1003–1016. [PubMed: 18004940]

Lambert-Messerlian GM, Pinar H, Laprade E, et al. Inhibins and activins in human fetal abnormalities. Mol Cell Endocrinol. 2004; 225(1–2):101–108. [PubMed: 15451574]

Lan Y, Ovitt CE, Cho ES, et al. Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. Development. 2004; 131(13):3207–3216. [PubMed: 15175245]

Lan Y, Ryan RC, Zhang Z, et al. Expression of Wnt9b and activation of canonical Wnt signaling during midfacial morphogenesis in mice. Dev Dyn. 2006; 235(5):1448–1454. [PubMed: 16496313]

Lancman JJ, Caruccio NC, Harfe BD, et al. Analysis of the regulation of lin-41 during chick and mouse limb development. Dev Dyn. 2005; 234(4):948–960. [PubMed: 16245339]

Lee CT, Risom T, Strauss WM, et al. MicroRNAs in mammalian development. Birth Defects Res C Embryo Today. 2006; 78(2):129–139. [PubMed: 16847889]

Lee Y, Jeon K, Lee JT, et al. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 2002; 21(17):4663–4670. [PubMed: 12198168]

Li X, Carthew RW. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell. 2005; 123(7):1267–1277. [PubMed: 16377567]

Lin EA, Kong L, Bai XH, et al. miR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1. J Biol Chem. 2009; 284(17):11326–11335. [PubMed: 19251704]

Liu W, Sun X, Braut A, et al. Distinct functions for Bmp signaling in lip and palate fusion in mice. Development. 2005; 132(6):1453–1461. [PubMed: 15716346]

Lu J, Qian J, Chen F, et al. Differential expression of components of the microRNA machinery during mouse organogenesis. Biochem Biophys Res Commun. 2005; 334(2):319–323. [PubMed: 16036130]

Maclean K, Smith J, St Heaps L, et al. Axenfeld-Rieger malformation and distinctive facial features: clues to a recognizable 6p25 microdeletion syndrome. Am J Med Genet A. 2005; 132(4):381–385. [PubMed: 15654696]

Martello G, Zacchigna L, Inui M, et al. MicroRNA control of Nodal signalling. Nature. 2007; 449(7159):183–188. [PubMed: 17728715]

Maschhoff KL, Anziano PQ, Ward P, et al. Conservation of Sox4 gene structure and expression during chicken embryogenesis. Gene. 2003; 320:23–30. [PubMed: 14597385]

Matsuda T, Yamamoto T, Muraguchi A, et al. Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3. J Biol Chem. 2001; 276(46):42908–42914. [PubMed: 11555647]

Matzuk MM, Kumar TR, Vassalli A, et al. Functional analysis of activins during mammalian development. Nature. 1995; 374(6520):354–356. [PubMed: 7885473]

McNamee CJ, Reed JE, Howard MR, et al. Promotion of neuronal cell adhesion by members of the IgLON family occurs in the absence of either support or modification of neurite outgrowth. J Neurochem. 2002; 80(6):941–948. [PubMed: 11953444]

Melnick M, Chen H, Buckley S, et al. Insulin-like growth factor II receptor, transforming growth factor-beta, and Cdk4 expression and the developmental epigenetics of mouse palate morphogenesis and dysmorphogenesis. Dev Dyn. 1998; 211(1):11–25. [PubMed: 9438420]

Mendelsohn C, Lohnes D, Decimo D, et al. Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development. 1994; 120(10):2749–2771. [PubMed: 7607068]

Mineno J, Okamoto S, Ando T, et al. The expression profile of microRNAs in mouse embryos. Nucleic Acids Res. 2006; 34(6):1765–1771. [PubMed: 16582102]

Minkoff R, Parker SB, Rundus VR, et al. Expression patterns of connexin43 protein during facial development in the chick embryo: associates with outgrowth, attachment, and closure of the midfacial primordia. Anat Rec. 1997; 248(2):279–290. [PubMed: 9185994]

Miska EA, Alvarez-Saavedra E, Townsend M, et al. Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. 2004; 5(9):R68. [PubMed: 15345052]

Mukhopadhyay P, Greene RM, Pisano MM, et al. Expression profiling of transforming growth factor beta superfamily genes in developing orofacial tissue. Birth Defects Res A Clin Mol Teratol. 2006; 76(7):528–543. [PubMed: 16933306]

Mukhopadhyay P, Greene RM, Zacharias W, et al. Developmental gene expression profiling of mammalian, fetal orofacial tissue. Birth Defects Res A Clin Mol Teratol. 2004; 70(12):912–926. [PubMed: 15578713]

Nakazawa M, Matsunaga K, Asamura S, et al. Molecular mechanisms of cleft lip formation in CL/Fr mice. Scand J Plast Reconstr Surg Hand Surg. 2008; 42(5):225–232. [PubMed: 18830900]

O'Rourke JR, Swanson MS, Harfe BD, et al. MicroRNAs in mammalian development and tumorigenesis. Birth Defects Res C Embryo Today. 2006; 78(2):172–179. [PubMed: 16847882]

Osoegawa K, Vessere GM, Utami KH, et al. Identification of novel candidate genes associated with cleft lip and palate using array comparative genomic hybridisation. J Med Genet. 2008; 45(2):81–86. [PubMed: 17873121]

Ovcharenko D, Kelnar K, Johnson C, et al. Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway. Cancer Res. 2007; 67(22):10782–10788. [PubMed: 18006822]

Pan Q, Chegini N. MicroRNA signature and regulatory functions in the endometrium during normal and disease states. Semin Reprod Med. 2008; 26(6):479–493. [PubMed: 18951330]

Pandey DP, Picard D. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. Mol Cell Biol. 2009; 29(13):3783–3790. [PubMed: 19414598]

Patel K, Connolly DJ, Amthor H, et al. Cloning and early dorsal axial expression of Flik, a chick follistatin-related gene: evidence for involvement in dorsalization/neural induction. Dev Biol. 1996; 178(2):327–342. [PubMed: 8812133]

Pilia G, Uda M, Macis D, et al. Jagged-1 mutation analysis in Italian Alagille syndrome patients. Hum Mutat. 1999; 14(5):394–400. [PubMed: 10533065]

Plasterk RH. Micro RNAs in animal development. Cell. 2006; 124(5):877–881. [PubMed: 16530032]

Pravtcheva DD, Wise TL. Igf2r improves the survival and transmission ratio of Igf2 transgenic mice. Mol Reprod Dev. 2008; 75(11):1678–1687. [PubMed: 18361416]

Rao Y, Lee Y, Jarjoura D, et al. A comparison of normalization techniques for microRNA microarray data. Stat Appl Genet Mol Biol. 2008; 7(1) Article 22.

Reinhart BJ, Slack FJ, Basson M, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000; 403(6772):901–906. [PubMed: 10706289]

Rice R, Spencer-Dene B, Connor EC, et al. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. J Clin Invest. 2004; 113(12):1692–1700. [PubMed: 15199404]

Richman JM, Tickle C. Epithelia are interchangeable between facial primordia of chick embryos and morphogenesis is controlled by the mesenchyme. Dev Biol. 1989; 136(1):201–210. [PubMed: 2806720]

Riley BM, Mansilla MA, Ma J, et al. Impaired FGF signaling contributes to cleft lip and palate. Proc Natl Acad Sci USA. 2007; 104(11):4512–4517. [PubMed: 17360555]

Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008; 18(10):505–516. [PubMed: 18774294]

Saetrom P, Snove O Jr, Rossi JJ. Epigenetics and microRNAs. Pediatr Res. 2007; 61(5 Pt 2):17R–23R.

Sansregret L, Goulet B, Harada R, et al. The p110 isoform of the CDP/Cux transcription factor accelerates entry into S phase. Mol Cell Biol. 2006; 26(6):2441–2455. [PubMed: 16508018]

Schofield J, Houzelstein D, Davies K, et al. Expression of the dystrophin-related protein (utrophin) gene during mouse embryogenesis. Dev Dyn. 1993; 198(4):254–264. [PubMed: 8130373]

Schutte BC, Murray JC. The many faces and factors of orofacial clefts. Hum Mol Genet. 1999; 8(10): 1853–1859. [PubMed: 10469837]

Smyth, G. Limma: linear models for microarray data. In: Gentleman, R.; Carey, V.; Dudoit, S.; Irizarry, R.; Huber, W., editors. Bioinformatics and computational biology solutions using R and Bioconductor. New York: Springer; 2005. p. 397-420.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004; 3 Article 3.

Smyth GK, Michaud J, Scott HS, et al. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics. 2005; 21(9):2067–2075. [PubMed: 15657102]

Sokol NS, Xu P, Jan YN, et al. Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis. Genes Dev. 2008; 22(12):1591–1596. [PubMed: 18559475]

Sommer P, Napier HR, Hogan BL, et al. Identification of Tgf beta1i4 as a downstream target of Foxc1. Dev Growth Differ. 2006; 48(5):297–308. [PubMed: 16759280]

Spritz RA. The genetics and epigenetics of orofacial clefts. Curr Opin Pediatr. 2001; 13(6):556–560. [PubMed: 11753106]

Sun D, Vanderburg CR, Odierna GS, et al. TGFbeta3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. Development. 1998; 125(1):95–105. [PubMed: 9389667]

Takada S, Berezikov E, Yamashita Y, et al. Mouse microRNA profiles determined with a new and sensitive cloning method. Nucleic Acids Res. 2006; 34(17):e115. [PubMed: 16973894]

Tatsuguchi M, Seok HY, Callis TE, et al. Expression of microRNAs is dynamically regulated during cardiomyocyte hypertrophy. J Mol Cell Cardiol. 2007; 42(6):1137–1141. [PubMed: 17498736]

Taya Y, O'Kane S, Ferguson MW, et al. Pathogenesis of cleft palate in TGF-beta3 knockout mice. Development. 1999; 126(17):3869–3879. [PubMed: 10433915]

Toyo-oka K, Hirotsune S, Gambello MJ, et al. Loss of the Max-interacting protein Mnt in mice results in decreased viability, defective embryonic growth and craniofacial defects: relevance to Miller-Dieker syndrome. Hum Mol Genet. 2004; 13(10):1057–1067. [PubMed: 15028671]

Troyanskaya O, Cantor M, Sherlock G, et al. Missing value estimation methods for DNA microarrays. Bioinformatics. 2001; 17(6):520–525. [PubMed: 11395428]

Van Buggenhout G, Van Ravenswaaij-Arts C, Mc Maas N, et al. The del(2)(q32.2q33) deletion syndrome defined by clinical and molecular characterization of four patients. Eur J Med Genet. 2005; 48(3):276–289. [PubMed: 16179223]

Voinnet O. Origin, biogenesis, and activity of plant microRNAs. Cell. 2009; 136(4):669–687. [PubMed: 19239888]

Wang LL, Zhang Z, Li Q, et al. Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. Hum Reprod. 2009; 24(3):562–579. [PubMed: 19091803]

Wang Y, Stricker HM, Gou D, et al. MicroRNA: past and present. Front Biosci. 2007; 12:2316–2329. [PubMed: 17127242]

Warner DR, Pisano MM, Greene RM, et al. Expression of the nuclear coactivators CBP and p300 in developing craniofacial tissue. In Vitro Cell Dev Biol Anim. 2002; 38(1):48–53. [PubMed: 11963968]

Weston WM, Potchinsky MB, Lafferty CM, et al. Cross-talk between signaling pathways in murine embryonic palate cells: effect of TGF beta and cAMP on EGF-induced DNA synthesis. In Vitro Cell Dev Biol Anim. 1998; 34(1):74–78. [PubMed: 9542639]

Wienholds E, Koudijs MJ, van Eeden FJ, et al. The microRNA-producing enzyme Dicer1 is essential for zebrafish development. Nat Genet. 2003; 35(3):217–218. [PubMed: 14528306]

Wu W, Lin Z, Zhuang Z, et al. Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. Eur J Cancer Prev. 2009; 18(1):50–55. [PubMed: 19077565]

Xu H, Wang X, Du Z, et al. Identification of microRNAs from different tissues of chicken embryo and adult chicken. FEBS Lett. 2006; 580(15):3610–3616. [PubMed: 16750530]

Xu X, Bringas P Jr, Soriano P, Chai Y. PDGFR-alpha signaling is critical for tooth cusp and palate morphogenesis. Dev Dyn. 2005; 232(1):75–84. [PubMed: 15543606]

Yang Q, Nagano T, Shah Y, et al. The PPAR alpha-humanized mouse: a model to investigate species differences in liver toxicity mediated by PPAR alpha. Toxicol Sci. 2008; 101(1):132–139. [PubMed: 17690133]

Yang WJ, Yang DD, Na S, et al. Dicer is required for embryonic angiogenesis during mouse development. J Biol Chem. 2005; 280(10):9330–9335. [PubMed: 15613470]

Yang Z, Wu J. Small RNAs and development. Med Sci Monit. 2006; 12(7):RA125–RA129. [PubMed: 16810144]

Yu F, Yao H, Zhu P, et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007; 131(6):1109–1123. [PubMed: 18083101]

Zhan M, Miller CP, Papayannopoulou T, et al. MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol. 2007; 35(7):1015–1025. [PubMed: 17588470]

Zhang B, Wang Q, Pan X, et al. MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol. 2007; 210(2):279–289. [PubMed: 17096367]

Zhao JJ, Sun DG, Wang J, et al. Retinoic acid downregulates micro-RNAs to induce abnormal development of spinal cord in spina bifida rat model. Childs Nerv Syst. 2008; 24(4):485–492. [PubMed: 17962954]

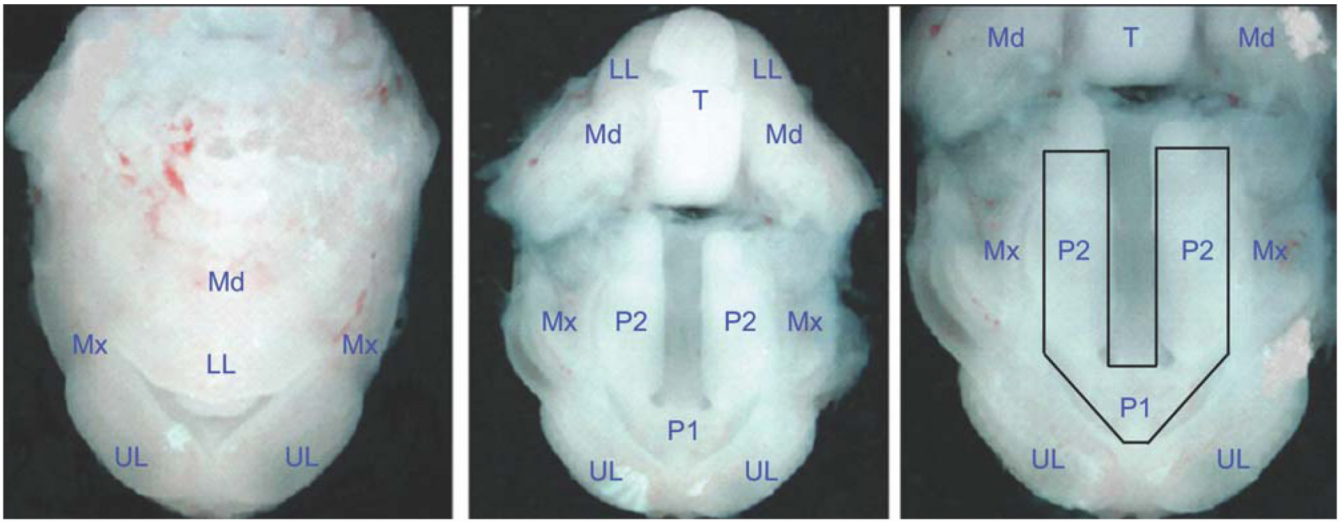

Figure 1.

Photomicrographs of ventral views of the developing orofacial region of a GD-13 mouse embryo. (A) Upper and lower lips and jaws (maxilla and mandible). (B) The embryonic oral cavity. The lower half of the photo contains the roof of the oral cavity with the maxillary processes, primary palate, and secondary palatal processes; the upper half contains the base/floor of the oral cavity showing the tongue and the mandible. (C) A magnified view of the roof of the oral cavity: note that the upper lip and the primary palate are completely formed, and the developing secondary palatal shelves are derived from the medial aspect of each maxillary process. The region demarcated by the black line was excised from GD-13 embryos for extraction of total RNA. Corresponding regions were dissected from the developing orofacial region of GD-12 and GD-14 embryos. UL = upper lip; LL = lower lip; Mx = maxilla; Md = mandible; P1 = primary palate; P2 = secondary palate; T = tongue. (Reprinted from Mukhopadhyay et al., 2006).

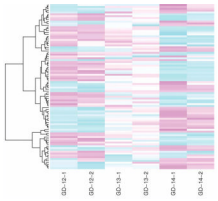

Figure 2.

Heat maps (hierarchical clusters) of differentially expressed miRNAs in developing murine orofacial tissue. Heat maps (hierarchical clusters) of all 90 miRNAs found to be significantly differentially expressed (adjusted p value < 0.05) for at least one of the three comparisons between gestational days (GD-13 vs. GD-12, GD-14 vs. GD-12, and GD-14 vs. GD-13; see Tables 2, 3, and 4, respectively) in developing murine orofacial tissue. Each row of the heat map represents a gene, and each column represents a time point in development (as labeled at the bottom; GD-12-1 = Gestation Day 12-sample replicate 1, etc.). Pink indicates an increase in miRNA gene expression (relative to the other expression measurements in the same row), whereas blue indicates a decrease.

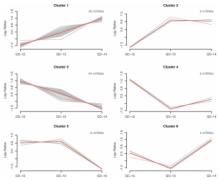

Figure 3.

Hierarchical cluster analysis illustrating six patterns of expression of microRNAs undergoing significant alteration in expression during murine orofacial development. Gene expression data sets from orofacial tissue from each of the critical days of orofacial development (GD-12, GD-13, and GD-14) were filtered and clustered as detailed in the Methods section. The solid red line on each graph represents the average miRNA expression pattern for the miRNA cluster, and the lighter gray lines of the graph represent the individual expression patterns for each miRNA. The expression pattern for each gene was normalized to mean 0 and SD 1 to better reflect the similarities between patterns based on the correlation measure (the measure used for clustering). The number above each graph indicates the number of miRNAs in the respective cluster. The lists of miRNA genes (90 genes) making up each cluster can be found in tabular form as supplementary material (Supplementary Table 4).

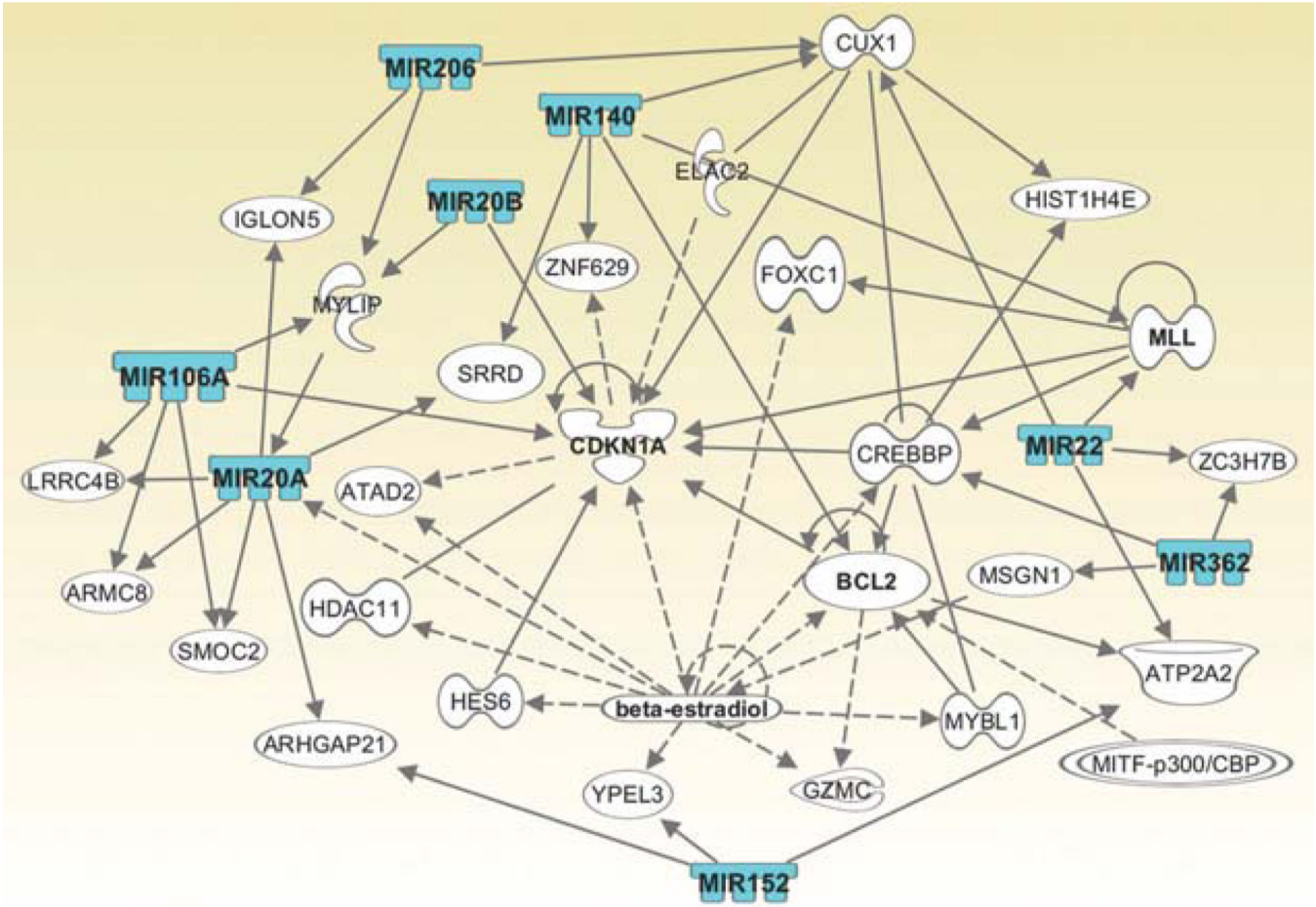

Figure 4.

Computational gene interaction predictions: selected microRNA gene network in developing orofacial tissue. A network with selected genes encoding microRNAs was constructed with IPA software. Several differentially regulated microRNA genes from the study were used to construct a gene association map as shown in this figure. Solid lines specify direct relationships, whereas dotted lines indicate indirect interactions.

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

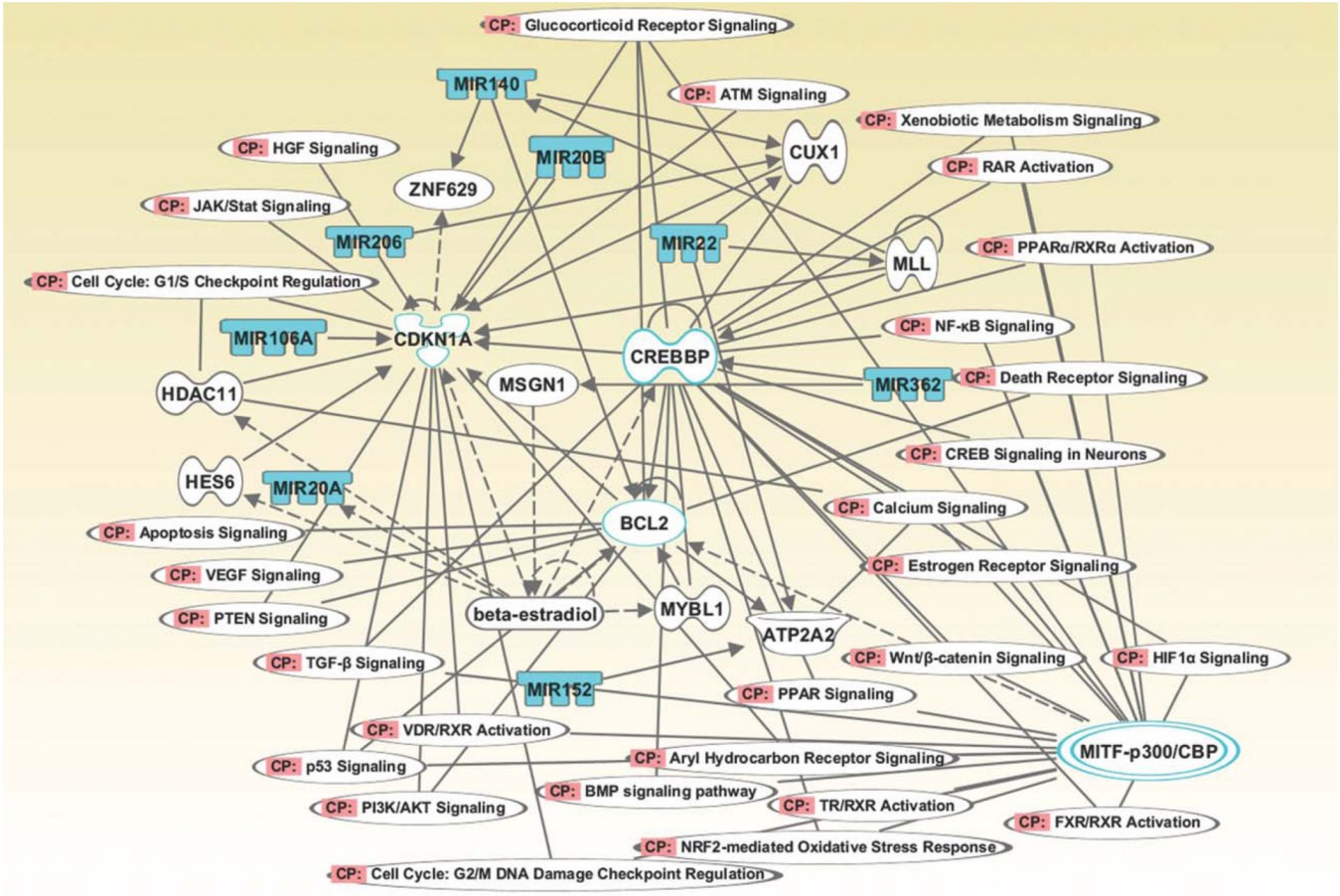

Figure 6.

Computational gene interaction predictions: selected microRNA gene network in the developing orofacial tissue. A network with selected genes encoding microRNAs was constructed with IPA software. Several differentially regulated microRNA genes from the study were used to construct a gene association map (Fig. 4) for highlighting diverse signaling pathways (as shown in this figure) operative within the developing mouse orofacial tissue. Solid lines specify direct relationships, whereas dotted lines indicate indirect interactions.

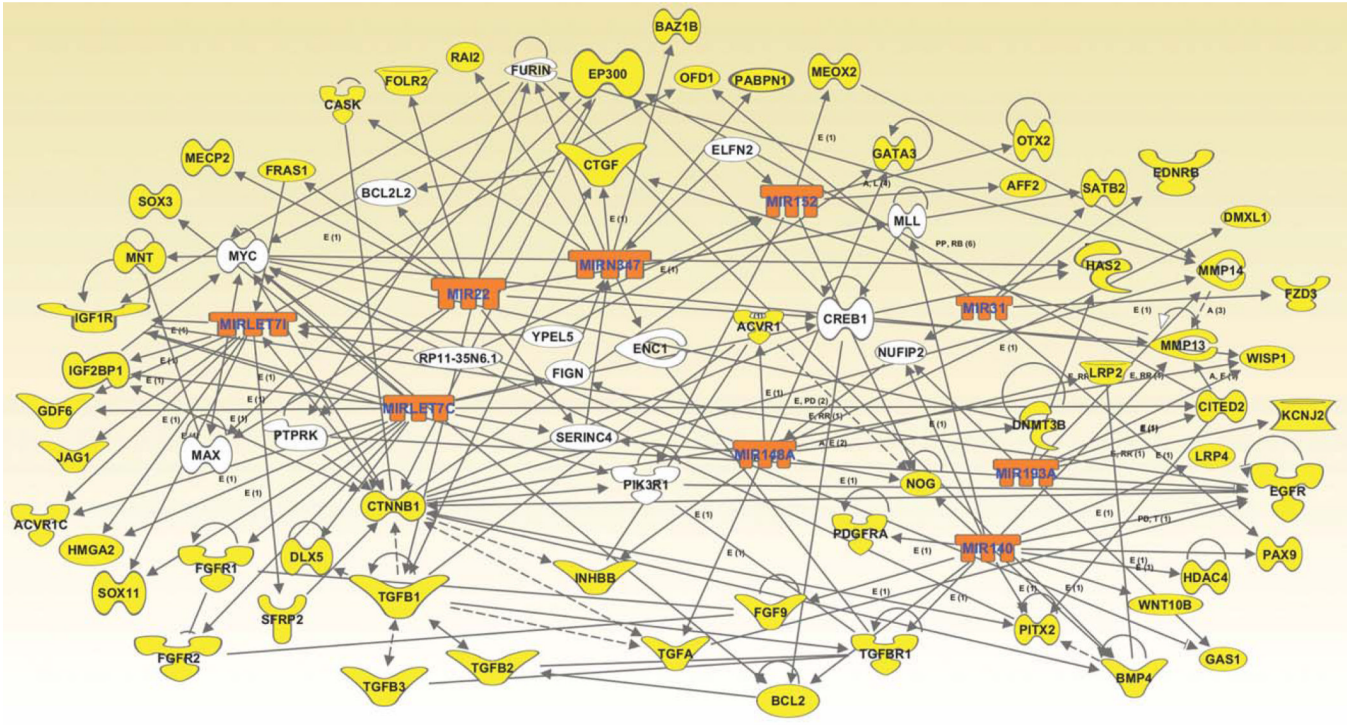

Figure 7.

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

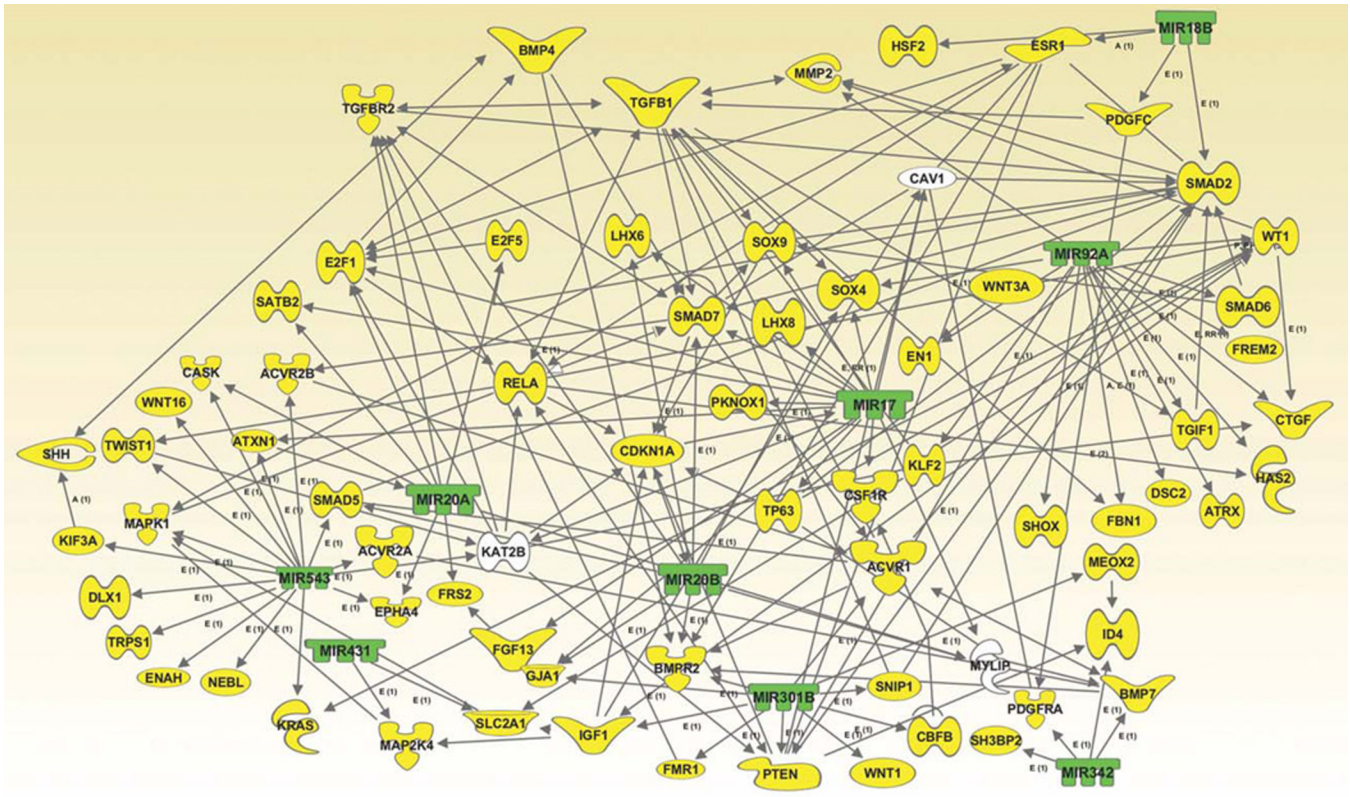

Figure 8.

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

Table 1

List of MicroRNAs Expressed in GD-12, GD-13, and GD-14 Orofacial Tissue

	miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
GD-12	*MIR-199A-3P-199B-3P: HSA-MIR-199A-3P, HSA-MIR-199B-3P, MMU-MIR-199A-3P, MMU-MIR-199B, RNO-MIR-199A-3P*	4699.1	4.24
	MIR-125B: HSA-MIR-125B, MMU-MIR-125B, RNO-MIR-125B	2086.3	3.83
	MIR-130A: additional species	621.1	3.70
	MIR-26B: HSA-MIR-26B, MMU-MIR-26B, RNO-MIR-26B	146.8	3.67
	MIR-99A: HSA-MIR-99A, MMU-MIR-99A, RNO-MIR-99A	355.1	3.63
	MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152	79.3	3.59
	MIR-19B: HSA-MIR-19B, MMU-MIR-19B, RNO-MIR-19B	1127.6	3.48
	MGHV-MIR-M1-2: MGHV-MIR-M1-2	68.9	3.34
	MIR-16: additional species	255.7	3.31
	MIR-181D: HSA-MIR-181D, MMU-MIR-181D, RNO-MIR-181D	177.9	3.30
	MIR-126-3P: HSA-MIR-126, MMU-MIR-126, RNO-MIR-126	186.0	3.26
	MIR-218: HSA-MIR-218, MMU-MIR-218, RNO-MIR-218	86.4	3.18
	MIR-181B: HSA-MIR-181B, MMU-MIR-181B, RNO-MIR-181B	211.1	3.08
	MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	538.7	3.05
	MIR-181A: HSA-MIR-181A, MMU-MIR-181A, RNO-MIR-181A	577.6	3.05
	MIR-335: HSA-MIR-335, MMU-MIR-335, RNO-MIR-335	490.9	3.04
	MIR-322-MIR-424: MMU-MIR-322, RNO-MIR-322, (MMU-MIR-424), (RNO-MIR-424)	128.2	3.01
	MIR-214: HSA-MIR-214, MMU-MIR-214, RNO-MIR-214	321.2	3.00
	MIR-130A: HSA-MIR-130A, MMU-MIR-130A, RNO-MIR-130A	1476.3	2.98
	MIR-205: HSA-MIR-205, MMU-MIR-205, RNO-MIR-205	540.0	2.94
	MIR-199B-5P: MMU-MIR-199B-5P (MMU-MIR-199B)*	1479.3	2.94
	MIR-30E: MMU-MIR-30E, RNO-MIR-30E, HSA-MIR-30E	58.0	2.59
	MIR-347: RNO-MIR-347	226.5	2.55
	MIR-19A: HSA-MIR-19A, MMU-MIR-19A, RNO-MIR-19A	193.1	2.44
	MIR-16: HSA-MIR-16, MMU-MIR-16, RNO-MIR-16	852.9	2.27
	MIR-26A: HSA-MIR-26A, MMU-MIR-26A, RNO-MIR-26A	957.2	2.19
	MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	186.2	2.17
	MIR-30B: HSA-MIR-30B, MMU-MIR-30B, RNO-MIR-30B	188.5	2.11
	MIR-15A: additional species	88.5	2.09
	MIR-21: HSA-MIR-21, MMU-MIR-21, RNO-MIR-21	75.1	2.08
	MIR-301: additional species	107.9	1.96
	MIR-92B: HSA-MIR-92B, MMU-MIR-92B, RNO-MIR-92B	73.5	1.95
	LET-7F: HSA-LET-7F, MMU-LET-7F, RNO-LET-7F	409.6	1.82
	LET-7I: HSA-LET-7I, MMU-LET-7I, RNO-LET-7I	185.0	1.82
	MIR-199B-5P: HSA-MIR-199B-5P	85.4	1.79
	MIR-20B: additional species	251.2	1.69
	MIR-148A: HSA-MIR-148A, MMU-MIR-148A	230.2	1.69
	MIR-199A-5P: HSA-MIR-199A-5P, MMU-MIR-199A-5P, RNO-MIR-199A-5P	2793.8	1.65

	miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
	MIR-93: HSA-MIR-93, MMU-MIR-93, RNO-MIR-93	243.2	1.55
	MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	156.0	1.52
	MIR-27B: HSA-MIR-27B, MMU-MIR-27B, RNO-MIR-27B	409.3	1.27
	MIR-106B: HSA-MIR-106B, MMU-MIR-106B, RNO-MIR-106B	495.6	1.20
	MIR-15B: additional species	210.8	1.19
	MIR-25: HSA-MIR-25, MMU-MIR-25, RNO-MIR-25	282.4	1.14
	MIR-106A: MMU-MIR-106A	379.3	1.09
	MIR-429: MMU-MIR-429, RNO-MIR-429	59.2	1.08
	MIR-376B: MMU-MIR-376B, RNO-MIR-376B	126.2	1.03
	MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	782.0	0.96
	MIR-451: HSA-MIR-451, MMU-MIR-451, RNO-MIR-451	107.0	0.95
	LET-7A: HSA-LET-7A, MMU-LET-7A, RNO-LET-7A	564.0	0.81
	MIR-106A: HSA-MIR-106A	913.0	0.80
	MIR-125A: HSA-MIR-125A, MMU-MIR-125A, RNO-MIR-125A	368.5	0.77
	LET-7G: HSA-LET-7G, MMU-LET-7G	268.1	0.75
	MIR-674: MMU-MIR-674, RNO-MIR-674-5P	57.1	0.72
	MIR-18A: HSA-MIR-18A, MMU-MIR-18A, RNO-MIR-18A	362.6	0.63
	MIR-200A: HSA-MIR-200A, MMU-MIR-200A, RNO-MIR-200A	95.7	0.63
	MIR-27A: HSA-MIR-27A, MMU-MIR-27A, RNO-MIR-27A	404.8	0.60
	MIR-744: HSA-MIR-744, MMU-MIR-744, RNO-MIR-744	52.2	0.53
	MIR-18B: HSA-MIR-18B	258.2	0.43
	MIR-15B: HSA-MIR-15B, MMU-MIR-15B, RNO-MIR-15B	269.0	0.38
	MIR-30A: HSA-MIR-30A, MMU-MIR-30A, RNO-MIR-30A	201.2	0.20
	MIR-130B: HSA-MIR-130B, MMU-MIR-130B, RNO-MIR-130B	264.7	0.17
	MIR-30C: HSA-MIR-30C, MMU-MIR-30C, RNO-MIR-30C	342.3	0.16
	MIR-24: HSA-MIR-24, MMU-MIR-24, RNO-MIR-24	554.9	0.16
	MIR-96: HSA-MIR-96, MMU-MIR-96, RNO-MIR-96	168.8	0.13
	MIR-99B: HSA-MIR-99B, MMU-MIR-99B, RNO-MIR-99B	317.5	0.13
	MIR-145: HSA-MIR-145, MMU-MIR-145, RNO-MIR-145	141.9	0.12
	MIR-301A: HSA-MIR-301A, MMU-MIR-301A, RNO-MIR-301A	732.8	0.08
GD-13	*MIR-411*: HSA-MIR-411*, MMU-MIR-411**	80.1	4.46
	MIR-199A-3P-199B-3P: HSA-MIR-199A-3P, HSA-MIR-199B-3P, MMU-MIR-199A-3P, MMU-MIR-199B, RNO-MIR-199A-3P	6963.8	4.23
	MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152	152.1	3.96
	MIR-125B: HSA-MIR-125B, MMU-MIR-125B, RNO-MIR-125B	3143.8	3.88
	MIR-26B: HSA-MIR-26B, MMU-MIR-26B, RNO-MIR-26B	247.3	3.78
	MIR-99A: HSA-MIR-99A, MMU-MIR-99A, RNO-MIR-99A	508.4	3.70
	MIR-130A: additional species	1009.0	3.58
	MIR-126-3P: HSA-MIR-126, MMU-MIR-126, RNO-MIR-126	361.6	3.43
	MIR-218: HSA-MIR-218, MMU-MIR-218, RNO-MIR-218	158.7	3.36
	MIR-16: additional species	450.1	3.34

miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
MIR-19B: HSA-MIR-19B, MMU-MIR-19B, RNO-MIR-19B	1557.9	3.28
MIR-181D: HSA-MIR-181D, MMU-MIR-181D, RNO-MIR-181D	229.2	3.19
MGHV-MIR-M1-2: MGHV-MIR-M1-2	97.1	3.17
MIR-322-MIR-424: MMU-MIR-322, RNO-MIR-322, (MMU-MIR-424), (RNO-MIR-424)	157.9	3.09
MIR-214: HSA-MIR-214, MMU-MIR-214, RNO-MIR-214	445.3	3.09
MIR-205: HSA-MIR-205, MMU-MIR-205, RNO-MIR-205	704.2	3.07
MIR-181A: HSA-MIR-181A, MMU-MIR-181A, RNO-MIR-181A	824.3	3.00
MIR-130A: HSA-MIR-130A, MMU-MIR-130A, RNO-MIR-130A	1826.6	3.00
MIR-181B: HSA-MIR-181B, MMU-MIR-181B, RNO-MIR-181B	266.1	2.98
MIR-199B-5P: MMU-MIR-199B-5P (MMU-MIR-199B)*	2111.4	2.93
MIR-347: RNO-MIR-347	347.0	2.78
MIR-335: HSA-MIR-335, MMU-MIR-335, RNO-MIR-335	780.8	2.78
MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	578.2	2.69
MIR-30E: MMU-MIR-30E, RNO-MIR-30E, HSA-MIR-30E	177.8	2.62
MIR-26A: HSA-MIR-26A, MMU-MIR-26A, RNO-MIR-26A	1712.2	2.35
MIR-16: HSA-MIR-16, MMU-MIR-16, RNO-MIR-16	1163.2	2.33
LET-7F: HSA-LET-7F, MMU-LET-7F, RNO-LET-7F	838.7	2.29
MIR-19A: HSA-MIR-19A, MMU-MIR-19A, RNO-MIR-19A	266.5	2.26
LET-7I: HSA-LET-7I, MMU-LET-7I, RNO-LET-7I	471.6	2.25
MIR-30B: HSA-MIR-30B, MMU-MIR-30B, RNO-MIR-30B	289.5	2.12
MIR-21: HSA-MIR-21, MMU-MIR-21, RNO-MIR-21	133.1	2.11
MIR-148A: HSA-MIR-148A, MMU-MIR-148A	573.5	2.10
MIR-15A: additional species	148.3	2.03
MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	203.1	1.96
MIR-199B-5P: HSA-MIR-199B-5P	166.8	1.91
MIR-199A-5P: HSA-MIR-199A-5P, MMU-MIR-199A-5P, RNO-MIR-199A-5P	3905.1	1.65
MIR-92B: HSA-MIR-92B, MMU-MIR-92B, RNO-MIR-92B	80.2	1.64
MIR-301: additional species	187.1	1.63
MIR-141: additional species	101.4	1.53
MIR-93: HSA-MIR-93, MMU-MIR-93, RNO-MIR-93	248.0	1.38
MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	178.9	1.26
MIR-20B: additional species	289.4	1.25
MIR-27B: HSA-MIR-27B, MMU-MIR-27B, RNO-MIR-27B	671.0	1.23
MIR-451: HSA-MIR-451, MMU-MIR-451, RNO-MIR-451	252.4	1.22
MIR-106B: HSA-MIR-106B, MMU-MIR-106B, RNO-MIR-106B	496.6	1.21
MIR-429: MMU-MIR-429, RNO-MIR-429	88.1	1.17
MIR-140-5P: HSA-MIR-140-5P, MMU-MIR-140, RNO-MIR-140	82.0	1.12
MIR-25: HSA-MIR-25, MMU-MIR-25, RNO-MIR-25	327.7	1.09
LET-7A: HSA-LET-7A, MMU-LET-7A, RNO-LET-7A	1095.9	1.05
LET-7G: HSA-LET-7G, MMU-LET-7G	518.4	1.05
MIR-15B: additional species	239.5	0.97

	miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
	MIR-125A: HSA-MIR-125A, MMU-MIR-125A, RNO-MIR-125A	585.3	0.87
	MIR-376B: MMU-MIR-376B, RNO-MIR-376B	205.5	0.86
	MIR-27A: HSA-MIR-27A, MMU-MIR-27A, RNO-MIR-27A	669.9	0.69
	MIR-106A: MMU-MIR-106A	512.1	0.68
	MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	909.6	0.60
	MIR-200A: HSA-MIR-200A, MMU-MIR-200A, RNO-MIR-200A	149.5	0.59
	MIR-106A: HSA-MIR-106A	934.1	0.53
	LET-7B: HSA-LET-7B, MMU-LET-7B, RNO-LET-7B	125.4	0.34
	LET-7C: HSA-LET-7C, MMU-LET-7C, RNO-LET-7C	563.1	0.33
	MIR-24: HSA-MIR-24, MMU-MIR-24, RNO-MIR-24	768.6	0.33
	MIR-30C: HSA-MIR-30C, MMU-MIR-30C, RNO-MIR-30C	474.3	0.29
	MIR-15B: HSA-MIR-15B, MMU-MIR-15B, RNO-MIR-15B	293.4	0.29
	MIR-145: HSA-MIR-145, MMU-MIR-145, RNO-MIR-145	243.1	0.25
	MIR-18A: HSA-MIR-18A, MMU-MIR-18A, RNO-MIR-18A	379.8	0.23
	MIR-96: HSA-MIR-96, MMU-MIR-96, RNO-MIR-96	224.9	0.23
	MIR-30A: HSA-MIR-30A, MMU-MIR-30A, RNO-MIR-30A	324.5	0.20
	MIR-744: HSA-MIR-744, MMU-MIR-744, RNO-MIR-744	88.1	0.20
	MIR-130B: HSA-MIR-130B, MMU-MIR-130B, RNO-MIR-130B	275.9	0.12
	LET-7E: HSA-LET-7E, MMU-LET-7E, RNO-LET-7E	527.2	0.09
	MIR-23A: HSA-MIR-23A, MMU-MIR-23A, RNO-MIR-23A	850.3	0.06
	MIR-99B: HSA-MIR-99B, MMU-MIR-99B, RNO-MIR-99B	425.3	0.02
GD-14	*MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152*	224.5	4.64
	MIR-199A-3P-199B-3P: HSA-MIR-199A-3P, HSA-MIR-199B-3P, MMU-MIR-199A-3P, MMU-MIR-199B, RNO-MIR-199A-3P	4808.8	4.18
	MIR-125B: HSA-MIR-125B, MMU-MIR-125B, RNO-MIR-125B	2356.6	3.98
	MIR-26B: HSA-MIR-26B, MMU-MIR-26B, RNO-MIR-26B	178.4	3.79
	MIR-99A: HSA-MIR-99A, MMU-MIR-99A, RNO-MIR-99A	476.5	3.65
	MIR-130A: additional species	647.9	3.57
	MIR-126-3P: HSA-MIR-126, MMU-MIR-126, RNO-MIR-126	252.9	3.54
	MIR-16: additional species	318.0	3.36
	MIR-218: HSA-MIR-218, MMU-MIR-218, RNO-MIR-218	137.1	3.25
	MIR-214: HSA-MIR-214, MMU-MIR-214, RNO-MIR-214	383.1	3.24
	MIR-181D: HSA-MIR-181D, MMU-MIR-181D, RNO-MIR-181D	157.1	3.07
	MIR-347: RNO-MIR-347	404.1	3.05
	MIR-181B: HSA-MIR-181B, MMU-MIR-181B, RNO-MIR-181B	211.5	2.98
	MIR-205: HSA-MIR-205, MMU-MIR-205, RNO-MIR-205	490.9	2.94
	MIR-335: HSA-MIR-335, MMU-MIR-335, RNO-MIR-335	864.2	2.84
	MIR-130A: HSA-MIR-130A, MMU-MIR-130A, RNO-MIR-130A	1287.6	2.82
	MIR-19B: HSA-MIR-19B, MMU-MIR-19B, RNO-MIR-19B	1238.6	2.80
	MIR-181A: HSA-MIR-181A, MMU-MIR-181A, RNO-MIR-181A	711.6	2.71
	MIR-26A: HSA-MIR-26A, MMU-MIR-26A, RNO-MIR-26A	1549.6	2.71

miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
MIR-30E: MMU-MIR-30E, RNO-MIR-30E, HSA-MIR-30E	104.6	2.58
MIR-199B-5P: MMU-MIR-199B-5P (MMU-MIR-199B)*	1600.0	2.46
MIR-30B: HSA-MIR-30B, MMU-MIR-30B, RNO-MIR-30B	238.2	2.40
LET-7I: HSA-LET-7I, MMU-LET-7I, RNO-LET-7I	423.8	2.39
MIR-322-MIR-424: MMU-MIR-322, RNO-MIR-322, (MMU-MIR-424), (RNO-MIR-424)	107.7	2.39
LET-7F: HSA-LET-7F, MMU-LET-7F, RNO-LET-7F	940.1	2.20
MIR-15A: additional species	129.3	2.08
MIR-16: HSA-MIR-16, MMU-MIR-16, RNO-MIR-16	947.5	2.03
MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	311.5	2.01
MIR-148A: HSA-MIR-148A, MMU-MIR-148A	559.0	2.00
MIR-451: HSA-MIR-451, MMU-MIR-451, RNO-MIR-451	554.8	2.00
MIR-21: HSA-MIR-21, MMU-MIR-21, RNO-MIR-21	155.9	1.97
MIR-141: additional species	94.2	1.88
MIR-19A: HSA-MIR-19A, MMU-MIR-19A, RNO-MIR-19A	218.7	1.80
MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	129.5	1.71
MIR-199B-5P: HSA-MIR-199B-5P	138.3	1.70
MIR-199A-5P: HSA-MIR-199A-5P, MMU-MIR-199A-5P, RNO-MIR-199A-5P	2691.5	1.58
MIR-301: additional species	146.0	1.41
MIR-27B: HSA-MIR-27B, MMU-MIR-27B, RNO-MIR-27B	561.3	1.32
MIR-429: MMU-MIR-429, RNO-MIR-429	85.8	1.23
LET-7A: HSA-LET-7A, MMU-LET-7A, RNO-LET-7A	1047.1	1.22
MIR-140-5P: HSA-MIR-140-5P, MMU-MIR-140, RNO-MIR-140	93.0	1.15
MIR-376B: MMU-MIR-376B, RNO-MIR-376B	185.7	1.13
LET-7G: HSA-LET-7G, MMU-LET-7G	523.2	1.11
LET-7B: HSA-LET-7B, MMU-LET-7B, RNO-LET-7B	186.9	1.05
MIR-93: HSA-MIR-93, MMU-MIR-93, RNO-MIR-93	158.6	1.04
MIR-25: HSA-MIR-25, MMU-MIR-25, RNO-MIR-25	223.6	0.96
MIR-20B: additional species	162.1	0.94
MIR-125A: HSA-MIR-125A, MMU-MIR-125A, RNO-MIR-125A	538.3	0.93
MIR-27A: HSA-MIR-27A, MMU-MIR-27A, RNO-MIR-27A	673.9	0.85
MIR-15B: additional species	146.9	0.75
MIR-106B: HSA-MIR-106B, MMU-MIR-106B, RNO-MIR-106B	311.4	0.75
LET-7C: HSA-LET-7C, MMU-LET-7C, RNO-LET-7C	669.3	0.73
MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	80.5	0.67
MIR-145: HSA-MIR-145, MMU-MIR-145, RNO-MIR-145	269.7	0.61
MIR-106A: MMU-MIR-106A	237.0	0.59
MIR-200A: HSA-MIR-200A, MMU-MIR-200A, RNO-MIR-200A	145.4	0.55
MIR-22: additional species	78.7	0.49
MIR-24: HSA-MIR-24, MMU-MIR-24, RNO-MIR-24	706.3	0.38
LET-7E: HSA-LET-7E, MMU-LET-7E, RNO-LET-7E	363.2	0.33
MIR-15B: HSA-MIR-15B, MMU-MIR-15B, RNO-MIR-15B	183.7	0.24

miRNA gene family: gene IDs	Expression (red)	Log ratio (red vs. green)
MIR-22: HSA-MIR-22, MMU-MIR-22, RNO-MIR-22	106.3	0.21
MIR-30C: HSA-MIR-30C, MMU-MIR-30C, RNO-MIR-30C	434.5	0.15
LET-7D: HSA-LET-7D, MMU-LET-7D, RNO-LET-7D	787.6	0.11
MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	470.3	0.10
MIR-30A: HSA-MIR-30A, MMU-MIR-30A, RNO-MIR-30A	321.4	0.06
MIR-23A: HSA-MIR-23A, MMU-MIR-23A, RNO-MIR-23A	801.2	0.01

Genes encoding miRNAs expressed in the developing orofacial tissue. The average expression signal value (red) corresponding to each gene was obtained by filtering the miRNA gene expression data sets from orofacial tissue from each of GD-12, GD-13, and GD-14 embryos. Criteria for considering an miRNA as expressed included a log2-ratio of red (experimental sample) vs. green (universal reference) intensity larger than zero, and a signal intensity in the experimental sample greater than or equal to the 50th percentile of the distribution.

Table 2

MicroRNAs Differentially Expressed in GD-13 versus GD-12 Orofacial Tissue

Day 13 versus 12: overexpressed				
miRNA gene family: gene IDs	**Fold change**[a]	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-193A-3P: HSA-MIR-193A-3P, MMU-MIR-193, RNO-MIR-193	4.03	6.15	0.000	0.000
MIR-193B: MMU-MIR-193B	1.81	4.89	0.000	0.002
LET-7B: HSA-LET-7B, MMU-LET-7B, RNO-LET-7B	1.75	8.28	0.000	0.000
MIR-31: HSA-MIR-31, MMU-MIR-31, RNO-MIR-31	1.63	4.59	0.000	0.003
MIR-140-5P: HSA-MIR-140-5P, MMU-MIR-140, RNO-MIR-140	1.48	5.74	0.000	0.001
LET-7C: HSA-LET-7C, MMU-LET-7C, RNO-LET-7C	1.47	5.41	0.000	0.001
LET-7I: HSA-LET-7I, MMU-LET-7I, RNO-LET-7I	1.35	4.45	0.000	0.004
MIR-210: HSA-MIR-210, MMU-MIR-210, RNO-MIR-210	1.34	3.23	0.004	0.031
MIR-148A: HSA-MIR-148A, MMU-MIR-148A	1.33	3.63	0.001	0.016
MIR-22: HSA-MIR-22, MMU-MIR-22, RNO-MIR-22	1.33	3.41	0.002	0.024
MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152	1.30	3.64	0.001	0.016
MIR-347: RNO-MIR-347	1.17	3.11	0.005	0.039
Day 13 versus 12: underexpressed				
MIR-342: additional species	0.53	−5.25	0.000	0.001
MIR-342-3P: HSA-MIR-342-3P, MMU-MIR-342-3P, RNO-MIR-342-3P	0.55	−4.31	0.000	0.005
*MIR-543-3P: HSA-MIR-543, MMU-MIR-543, RNO-MIR-543**	0.61	−5.10	0.000	0.001
MIR-301B: HSA-MIR-301B	0.71	−4.04	0.001	0.007
MIR-298: MMU-MIR-298, RNO-MIR-298	0.72	−4.13	0.000	0.006
MIR-18B: HSA-MIR-18B	0.73	−3.24	0.004	0.031
MIR-20B: additional species	0.73	−5.26	0.000	0.001
MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	0.78	−4.11	0.000	0.006
MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	0.78	−3.27	0.003	0.030
MIR-301: additional species	0.80	−4.22	0.000	0.005
MIR-431: HSA-MIR-431, MMU-MIR-431, RNO-MIR-431	0.81	−3.48	0.002	0.022
MIR-92B: HSA-MIR-92B, MMU-MIR-92B, RNO-MIR-92B	0.81	−3.06	0.006	0.043
MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	0.84	−3.27	0.003	0.030
MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	0.87	−3.41	0.002	0.024

[a] Gene expression from orofacial tissue from each of GD-12, GD-13, and GD-14 embryos were filtered, and the average fold change for each gene was calculated for GD-13 vs. GD-12, GD-14 vs. GD-12, and GD-14 vs. GD-13 orofacial tissue. Only those genes that demonstrated a statistically significant (adjusted $p < 0.05$) increase or decrease in expression for the GD-13 vs. GD-12 expression comparison were included in this table. Note that GD-13 vs. GD-12 means that expression on gestation day 12 was utilized as the baseline. Therefore, ratios below 1.0 indicate a decrease in expression, whereas ratios above 1.0 indicate an increase in expression.

Table 3

MicroRNAs Differentially Expressed in GD-14 versus GD-12 Orofacial Tissue

Day 14 versus 12: overexpressed				
miRNA gene family: gene IDs	**Fold change[a]**	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-193A-3P: HSA-MIR-193A-3P, MMU-MIR-193, RNO-MIR-193	6.48	8.25	0.000	0.000
MIR-352: RNO-MIR-352	5.24	2.69	0.013	0.033
MIR-206: HSA-MIR-206, MMU-MIR-206, RNO-MIR-206	3.93	3.41	0.002	0.008
MIR-98: HSA-MIR-98, MMU-MIR-98, RNO-MIR-98	3.01	3.15	0.005	0.014
LET-7B: HSA-LET-7B, MMU-LET-7B, RNO-LET-7B	2.87	15.51	0.000	0.000
MIR-154: HSA-MIR-154, MMU-MIR-154, RNO-MIR-154	2.78	4.58	0.000	0.001
MIR-133A: HSA-MIR-133A, MMU-MIR-133A, RNO-MIR-133A	2.70	4.40	0.000	0.001
MIR-422A: HSA-MIR-422A	2.67	4.54	0.000	0.001
MIR-22: HSA-MIR-22, MMU-MIR-22, RNO-MIR-22	2.61	11.47	0.000	0.000
MIR-133B: HSA-MIR-133B, MMU-MIR-133B, RNO-MIR-133B	2.50	3.59	0.002	0.005
MIR-193B: MMU-MIR-193B	2.49	7.57	0.000	0.000
MIR-22: additional species	2.33	3.00	0.006	0.019
MIR-451: HSA-MIR-451, MMU-MIR-451, RNO-MIR-451	2.08	7.70	0.000	0.000
MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152	2.07	10.25	0.000	0.000
LET-7C: HSA-LET-7C, MMU-LET-7C, RNO-LET-7C	1.93	9.31	0.000	0.000
MIR-141: HSA-MIR-141, MMU-MIR-141, RNO-MIR-141	1.91	6.04	0.000	0.000
LET-7D: HSA-LET-7D, MMU-LET-7D, RNO-LET-7D	1.89	4.92	0.000	0.000
MIR-210: HSA-MIR-210, MMU-MIR-210, RNO-MIR-210	1.72	5.95	0.000	0.000
MIR-31: HSA-MIR-31, MMU-MIR-31, RNO-MIR-31	1.72	4.99	0.000	0.000
MIR-351: RNO-MIR-351, MMU-MIR-351	1.68	4.30	0.000	0.001
MIR-140-5P: HSA-MIR-140-5P, MMU-MIR-140, RNO-MIR-140	1.52	6.09	0.000	0.000
MIR-127: HSA-MIR-127, MMU-MIR-127, RNO-MIR-127	1.51	3.79	0.001	0.004
MIR-141: additional species	1.50	6.56	0.000	0.000
LET-7I: HSA-LET-7I, MMU-LET-7I, RNO-LET-7I	1.50	5.92	0.000	0.000
MIR-100: HSA-MIR-100, MMU-MIR-100, RNO-MIR-100	1.50	4.70	0.000	0.001
MIR-434-3P: MMU-MIR-434-3P, RNO-MIR-434	1.47	4.08	0.000	0.002
MIR-26A: HSA-MIR-26A, MMU-MIR-26A, RNO-MIR-26A	1.43	3.78	0.001	0.004
MIR-347: RNO-MIR-347	1.42	6.88	0.000	0.000
MIR-503: MMU-MIR-503, RNO-MIR-503	1.41	3.21	0.004	0.012
MIR-145: HSA-MIR-145, MMU-MIR-145, RNO-MIR-145	1.41	4.11	0.000	0.002
MIR-191: HSA-MIR-191, MMU-MIR-191, RNO-MIR-191	1.35	2.94	0.007	0.020
MIR-503: HSA-MIR-503	1.27	2.94	0.007	0.021
MIR-381: HSA-MIR-381, MMU-MIR-381, RNO-MIR-381	1.25	2.90	0.008	0.022
MIR-494: HSA-MIR-494, MMU-MIR-494, RNO-MIR-494	1.24	2.57	0.017	0.041
MIR-148A: HSA-MIR-148A, MMU-MIR-148A	1.24	2.75	0.011	0.029
MIR-30B: HSA-MIR-30B, MMU-MIR-30B, RNO-MIR-30B	1.22	3.45	0.002	0.007
MIR-126-3P: HSA-MIR-126, MMU-MIR-126, RNO-MIR-126	1.21	3.21	0.004	0.012
MIR-143: HSA-MIR-143, MMU-MIR-143, RNO-MIR-143	1.20	2.56	0.017	0.041

Day 14 versus 12: overexpressed				
miRNA gene family: gene IDs	**Fold change[a]**	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-24: HSA-MIR-24, MMU-MIR-24, RNO-MIR-24	1.16	2.57	0.017	0.041
Day 14 versus 12: underexpressed				
MIR-1224-3P: HSA-MIR-1224-3P	0.25	−3.95	0.001	0.003
MIR-940: HSA-MIR-940	0.31	−2.64	0.015	0.036
MIR-362-5P: HSA-MIR-362-5P	0.34	−3.05	0.006	0.017
MIR-362-5P: MMU-MIR-362-5P, RNO-MIR-362-5P	0.34	−3.94	0.001	0.003
MIR-423-3P: HSA-MIR-423-3P, MMU-MIR-423-3P, RNO-MIR-423	0.40	−4.17	0.000	0.002
MIR-301B: HSA-MIR-301B	0.42	−9.96	0.000	0.000
MIR-106A: HSA-MIR-106A	0.48	−8.86	0.000	0.000
MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	0.48	−11.73	0.000	0.000
MIR-369-5P: HSA-MIR-369-5P, MMU-MIR-369-5P, RNO-MIR-369-5P	0.49	−2.98	0.007	0.019
MIR-18A: HSA-MIR-18A, MMU-MIR-18A, RNO-MIR-18A	0.49	−7.44	0.000	0.000
MIR-301A: HSA-MIR-301A, MMU-MIR-301A, RNO-MIR-301A	0.50	−3.53	0.002	0.006
MIR-203: HSA-MIR-203, MMU-MIR-203, RNO-MIR-203	0.51	−3.03	0.006	0.018
MIR-18B: HSA-MIR-18B	0.51	−6.84	0.000	0.000
*MIR-543-3P: HSA-MIR-543, MMU-MIR-543, RNO-MIR-543**	0.52	−6.77	0.000	0.000
MIR-350: MMU-MIR-350, RNO-MIR-350	0.53	−3.64	0.001	0.005
MIR-298: MMU-MIR-298, RNO-MIR-298	0.55	−7.68	0.000	0.000
MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	0.55	−7.86	0.000	0.000
MGHV-MIR-M1-2: MGHV-MIR-M1-2	0.56	−5.71	0.000	0.000
MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	0.56	−10.78	0.000	0.000
MIR-92B: HSA-MIR-92B, MMU-MIR-92B, RNO-MIR-92B	0.58	−7.91	0.000	0.000
MIR-20B: additional species	0.59	−8.87	0.000	0.000
MIR-30E: HSA-MIR-30E*, MMU-MIR-30E*, RNO-MIR-30E**	0.60	−5.37	0.000	0.000
MIR-342: additional species	0.61	−4.12	0.000	0.002
MIR-18B: MMU-MIR-18B, RNO-MIR-18B	0.61	−3.95	0.001	0.003
MIR-19B: HSA-MIR-19B, MMU-MIR-19B, RNO-MIR-19B	0.62	−8.02	0.000	0.000
MIR-19A: HSA-MIR-19A, MMU-MIR-19A, RNO-MIR-19A	0.64	−9.42	0.000	0.000
MIR-322-MIR-424: MMU-MIR-322, RNO-MIR-322, (MMU-MIR-424), (RNO-MIR-424)	0.65	−6.10	0.000	0.000
MIR-410: HSA-MIR-410, MMU-MIR-410, RNO-MIR-410	0.67	−5.49	0.000	0.000
MIR-301: additional species	0.68	−7.02	0.000	0.000
MIR-199B-5P: MMU-MIR-199B-5P (MMU-MIR-199B)*	0.70	−2.71	0.013	0.032
MIR-301B: MMU-MIR-301B, RNO-MIR-301B	0.70	−5.00	0.000	0.000
MIR-93: HSA-MIR-93, MMU-MIR-93, RNO-MIR-93	0.71	−5.50	0.000	0.000
MIR-106A: MMU-MIR-106A	0.71	−2.49	0.020	0.047
MIR-342-3P: HSA-MIR-342-3P, MMU-MIR-342-3P, RNO-MIR-342-3P	0.71	−2.49	0.020	0.047
MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	0.73	−7.55	0.000	0.000
MIR-106B: HSA-MIR-106B, MMU-MIR-106B, RNO-MIR-106B	0.73	−3.71	0.001	0.004
MIR-744: HSA-MIR-744, MMU-MIR-744, RNO-MIR-744	0.74	−2.86	0.009	0.023
MIR-15B: additional species	0.74	−4.26	0.000	0.001

Day 14 versus 12: overexpressed				
miRNA gene family: gene IDs	**Fold change**[a]	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-674: MMU-MIR-674, RNO-MIR-674-5P	0.75	−3.89	0.001	0.003
MIR-361-5P: HSA-MIR-361-5P, MMU-MIR-361, RNO-MIR-361	0.77	−3.21	0.004	0.012
MIR-652: HSA-MIR-652, MMU-MIR-652, RNO-MIR-652	0.78	−3.62	0.001	0.005
MIR-409-3P: HSA-MIR-409-3P, RNO-MIR-409-3P, MMU-MIR-409-3P	0.78	−2.90	0.008	0.022
MIR-431: HSA-MIR-431, MMU-MIR-431, RNO-MIR-431	0.79	−3.90	0.001	0.003
MIR-495: HSA-MIR-495, MMU-MIR-495, RNO-MIR-495	0.79	−2.70	0.013	0.032
MIR-181A: HSA-MIR-181A, MMU-MIR-181A, RNO-MIR-181A	0.79	−3.55	0.002	0.006
MIR-708: HSA-MIR-708, MMU-MIR-708, RNO-MIR-708	0.80	−2.96	0.007	0.020
MIR-181D: HSA-MIR-181D, MMU-MIR-181D, RNO-MIR-181D	0.86	−2.87	0.009	0.023

[a]Gene expression from orofacial tissue from each of GD-12, GD-13, and GD-14 embryos were filtered, and the average fold change for each gene was calculated for GD-13 vs. GD-12, GD-14 vs. GD-12, and GD-14 vs. GD-13 orofacial tissue. Only those genes that demonstrated a statistically significant (adjusted $p < 0.05$) increase or decrease in expression for the GD-14 vs. GD-12 expression comparison were included in this table. Note that GD-14 vs. GD-12 means that expression on gestation day 12 was utilized as the baseline. Therefore, ratios below 1.0 indicate a decrease in expression, whereas ratios above 1.0 indicate an increase in expression.

Table 4

MicroRNAs Differentially Expressed in GD-14 versus GD-13 Orofacial Tissue

Day 14 versus 13: overexpressed				
miRNA gene family: gene IDs	**Fold change[a]**	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-188-3P: HSA-MIR-188-3P, MMU-MIR-188-3P, RNO-MIR-188-3P	3.55	3.34	0.003	0.020
MIR-22: additional species	2.51	3.30	0.003	0.020
MIR-22: HSA-MIR-22, MMU-MIR-22, RNO-MIR-22	1.96	8.06	0.000	0.000
MIR-133A: HSA-MIR-133A, MMU-MIR-133A, RNO-MIR-133A	1.94	2.93	0.008	0.039
MIR-154: HSA-MIR-154, MMU-MIR-154, RNO-MIR-154	1.92	2.92	0.008	0.039
MIR-212: HSA-MIR-212, MMU-MIR-212, RNO-MIR-212	1.89	3.32	0.003	0.020
MIR-451: HSA-MIR-451, MMU-MIR-451, RNO-MIR-451	1.72	5.71	0.000	0.000
LET-7B: HSA-LET-7B, MMU-LET-7B, RNO-LET-7B	1.63	7.24	0.000	0.000
MIR-152: HSA-MIR-152, MMU-MIR-152, RNO-MIR-152	1.60	6.52	0.000	0.000
MIR-494: HSA-MIR-494, MMU-MIR-494, RNO-MIR-494	1.58	5.44	0.000	0.000
LET-7D: HSA-LET-7D, MMU-LET-7D, RNO-LET-7D	1.49	3.03	0.006	0.034
MIR-141: HSA-MIR-141, MMU-MIR-141, RNO-MIR-141	1.44	3.40	0.002	0.019
LET-7C: HSA-LET-7C, MMU-LET-7C, RNO-LET-7C	1.32	3.90	0.001	0.008
MIR-145: HSA-MIR-145, MMU-MIR-145, RNO-MIR-145	1.28	3.02	0.006	0.034
MIR-141: additional species	1.27	3.82	0.001	0.009
MIR-30B: HSA-MIR-30B, MMU-MIR-30B, RNO-MIR-30B	1.21	3.33	0.003	0.020
MIR-347: RNO-MIR-347	1.21	3.71	0.001	0.010
MIR-376B: MMU-MIR-376B, RNO-MIR-376B	1.21	2.94	0.007	0.039
Day 14 versus 13: underexpressed				
MIR-106A: HSA-MIR-106A	0.58	−6.62	0.000	0.000
MIR-301B: HSA-MIR-301B	0.60	−5.91	0.000	0.000
MIR-322-MIR-424: MMU-MIR-322, RNO-MIR-322, (MMU-MIR-424), (RNO-MIR-424)	0.61	−6.87	0.000	0.000
MIR-20A: HSA-MIR-20A, MMU-MIR-20A, RNO-MIR-20A	0.62	−7.62	0.000	0.000
MGHV-MIR-M1-2: MGHV-MIR-M1-2	0.63	−4.52	0.000	0.002
MIR-18A: HSA-MIR-18A, MMU-MIR-18A, RNO-MIR-18A	0.65	−4.52	0.000	0.002
MIR-20B: HSA-MIR-20B, MMU-MIR-20B, RNO-MIR-20B-5P	0.67	−7.50	0.000	0.000
MIR-18B: HSA-MIR-18B	0.70	−3.60	0.002	0.013
MIR-17: HSA-MIR-17, MMU-MIR-17, RNO-MIR-17 (RNO-MIR-17-5P)	0.71	−4.59	0.000	0.002
MIR-92B: HSA-MIR-92B, MMU-MIR-92B, RNO-MIR-92B	0.71	−4.85	0.000	0.001
MIR-19B: HSA-MIR-19B, MMU-MIR-19B, RNO-MIR-19B	0.72	−5.65	0.000	0.000
MIR-30E: HSA-MIR-30E*, MMU-MIR-30E*, RNO-MIR-30E**	0.72	−3.38	0.003	0.019
MIR-106B: HSA-MIR-106B, MMU-MIR-106B, RNO-MIR-106B	0.72	−3.82	0.001	0.009
MIR-19A: HSA-MIR-19A, MMU-MIR-19A, RNO-MIR-19A	0.73	−6.84	0.000	0.000
MIR-96: HSA-MIR-96, MMU-MIR-96, RNO-MIR-96	0.74	−3.11	0.005	0.030
MIR-298: MMU-MIR-298, RNO-MIR-298	0.76	−3.48	0.002	0.016
MIR-93: HSA-MIR-93, MMU-MIR-93, RNO-MIR-93	0.79	−3.74	0.001	0.010
MIR-652: HSA-MIR-652, MMU-MIR-652, RNO-MIR-652	0.80	−3.25	0.004	0.022

Day 14 versus 13: overexpressed				
miRNA gene family: gene IDs	**Fold change**[a]	***t* statistic**	***p* value**	**Adjusted *p* value**
MIR-20B: additional species	0.81	−3.60	0.002	0.013
MIR-181A: HSA-MIR-181A, MMU-MIR-181A, RNO-MIR-181A	0.82	−3.08	0.005	0.031
MIR-301B: MMU-MIR-301B, RNO-MIR-301B	0.82	−2.91	0.008	0.039
MIR-410: HSA-MIR-410, MMU-MIR-410, RNO-MIR-410	0.82	−2.79	0.010	0.049
MIR-92A: HSA-MIR-92A, MMU-MIR-92A, RNO-MIR-92A	0.84	−4.14	0.000	0.005
MIR-301: additional species	0.86	−2.80	0.010	0.049

[a]Gene expression from orofacial tissue from each of GD-12, GD-13, and GD-14 embryos were filtered, and the average fold change for each gene was calculated for GD-13 vs. GD-12, GD-14 vs. GD-12, and GD-14 vs. GD-13 orofacial tissue. Only those genes that demonstrated a statistically significant (adjusted $p < 0.05$) increase or decrease in expression for the GD-14 vs. GD-13 expression comparison were included in this table. Note that GD-14 vs. GD-13 means that expression on gestation day 13 was utilized as the baseline. Therefore, ratios below 1.0 indicate a decrease in expression, whereas ratios above 1.0 indicate an increase in expression.

Table 5

TaqMan Verification of Differentially Expressed Genes Encoding miRNAs in the Developing Mouse Orofacial Tissue

	Microarray		TaqMan QRTPCR		
Gene name[a]	**FC 13 vs. 12**	**FC 14 vs. 13**	**FC 13 vs. 12**	**FC 14 vs. 13**	**Concordance[b]**
HSA-MIR-193A-3P, MMU-MIR-193	+4.03	+1.60	+2.06	+1.87	+/+
MIR-193B: MMU-MIR-193B	+1.80	+1.38	+1.72	+1.38	+/+
HSA-LET-7B, MMU-LET-7B	+1.75	+1.63	+3.73	+3.07	+/+
HSA-MIR-22, MMU-MIR-22	+1.33	+1.96	+2.38	+3.24	+/+
HSA-MIR-152, MMU-MIR-152	+1.30	+1.60	+1.64	+2.73	+/+
HSA-MIR-206, MMU-MIR-206	+1.70	+2.30	+2.35	+5.10	+/+
HSA-MIR-20B, MMU-MIR-20B	−1.20	−1.50	−1.08	−1.44	+/+
HSA-MIR-342-3P, MMU-MIR-342-3P	−1.80	+1.27	−1.08	+1.62	+/+
HSA-MIR-1224-3p	−1.84	−2.20	−1.17	−2.58	+/+
HSA-MIR-362-5p	−1.58	−1.88	−1.87	+3.27	−/+
HSA-MIR-106A	−1.26	−1.73	−1.31	+1.52	−/+
HSA-MIR-140, MMU-MIR-140	+1.48	+1.02	+1.80	+2.50	+/+
HSA-MIR-20A, MMU-MIR-20A	−1.30	−1.60	−1.23	−1.35	+/+

Differential expression of genes encoding miRNAs in murine fetal orofacial tissue samples (GD-12, GD-13, and GD-14). Gene expression was compared using Miltenyi miRexplore miRNA arrays and TaqMan quantitative real-time PCR as detailed in Materials and Methods.

[a] Target genes encoding miRNAs were selected based on results from Miltenyi miRexplore miRNA arrays.

[b] +/+ Indicates full concordance between the pattern/level of gene expression from Miltenyi miRexplore miRNA arrays and TaqMan quantitative real-time PCR.

Table 6

Partial List of Target Genes (of Selected Differentially Expressed miRNAs) Known to Be Either Associated with, or Indispensable for, Mammalian Orofacial Ontogenesis

miRNA type[a]	Target gene[b]	Gene name	Score[c]
mmu-miR-140	*Pitx2*	Paired-like homeodomain transcription factor 2	93
mmu-miR-152	*Meox2*	Mesenchyme homeobox 2	92
mmu-miR-140	*Fgf9*	Fibroblast growth factor 9	92
hsa-miR-1224-3p	*Crebl1*	cAMP responsive element-binding protein-like 1	88
mmu-miR-20b	*Creb1*	cAMP responsive element-binding protein 1	84
mmu-miR-206	*Meox2*	Mesenchyme homeobox 2	83
mmu-miR-20b	*Tgfbr2*	TGFβ receptor II	82
mmu-miR-140	*Frs2*	Fibroblast growth factor receptor substrate 2	78
mmu-miR-206	*Frs2*	Fibroblast growth factor receptor substrate 2	78
mmu-miR-22	*Pdap1*	PDGFA-associated protein 1	77
mmu-miR-20a	*Smad7*	MAD homolog 7 (Drosophila)	76
mmu-miR-20b	*Smad7*	MAD homolog 7 (Drosophila)	76
mmu-miR-20a	*Tgfbr2*	TGFβ receptor II	76
mmu-miR-152	*Noggin*	Noggin	76
mmu-miR-20b	*Bambi*	BMP and Activin membrane-bound inhibitor, homolog	74
mmu-miR-140	*Pdgfra*	Platelet-derived growth factor receptor, alpha polypeptide	72
mmu-miR-152	*Wnt10b*	Wingless-related MMTV integration site 10b	72
hsa-miR-362-5p	*Acvr2A*	Activin A receptor, type IIA	72
mmu-let-7b	*Tgfbr3*	TGFβ receptor III	71
mmu-let-7b	*Acvr1c*	Activin A receptor, type IC	71
mmu-miR-140	*Creb3l1*	cAMP responsive element-binding protein 3-like 1	70
mmu-miR-140	*Tgfbr1*	TGFβ receptor I	69
hsa-miR-1224-3p	*Tgif2*	TGFβ-induced factor homeobox 2	67
hsa-miR-362-5p	*Acvrl1*	Activin A receptor type II-like 1	67
mmu-miR-20a	*Bambi*	BMP and Activin membrane-bound inhibitor, homolog	67
mmu-miR-342-3p	*Pdgfra*	Platelet-derived growth factor receptor, alpha polypeptide	66
mmu-miR-342-3p	*Wnt3*	Wingless-related MMTV integration site 3	66
mmu-miR-20b	*Bmpr2*	BMP receptor II	66
hsa-miR-1224-3p	*Pdgfδ*	Platelet-derived growth factor δ	65
mmu-miR-20a	*Lhx6*	LIM homeobox protein 6	65
mmu-miR-20b	*Lhx6*	LIM homeobox protein 6	65
mmu-miR-20a	*Creb1*	cAMP responsive element-binding protein 1	65
mmu-miR-20b	*Lhx8*	LIM homeobox protein 8	64
hsa-miR-1224-3p	*Crebzf*	CREB/ATF bZIP transcription factor	64
mmu-miR-206	*Cited2*	Cbp/p300-interacting transactivator, with Glu/Asp-rich c-t-dm2	63
mmu-miR-20b	*Pdgfrα*	Platelet-derived growth factor receptor, alpha polypeptide	60
mmu-miR-20a	*Bmpr2*	BMP receptor II	60

[a] Differential expression of these miRNAs during orofacial development (GD-12–GD-14) has been verified by TaqMan QRTPCR (see Table 5).

[b] Target analysis of these miRNAs was performed using the miRDB (http://mirdb.org/miRDB/) [52], as described in the Methods section

[c] miRNA target scoring was done as described in the Methods section.

Table 7

Partial List of Target Genes (of Selected Differentially Expressed miRNAs) Known or Likely to Be Associated with Biological (Cellular) Processes Indispensable for Mammalian Orofacial Ontogenesis

Target genes[a,b]	Cellular process[c,d]
Timp2, Frk, Rb1, Tns3, Smarcb1, Mfn2, SKAP2, Bcl2l2, Cnbp, Uhrf2, SUZ12, Mycn, PDGFD, Bdnf, Mitf, Pdcd1lg2, Topors, Pbx1, CDC7, Pitx2, Mab21l1, Frs2, Lhx9, Cxcr4, Acvr1c, Id4, Vegfa, Pten, Kit, Tsg101, Epgn, Acvr2a, Tgfbr2, Igf1, Hoxd13, Mecp2, Ereg, Edn1, Cspg4, Derl2, Zfp91, Ptprc, DBH, Cav2, Col4a3, Nras, Birc6, Cdkn1a, Lgr4, Fgf9, A730098P11Rik, SPRY2, Sema5a, Epha7, Pitx2, Smad2, Smad5,	Proliferation
Adamts15, Col2a1,Timp2, Smoc2, Gpc2, Wnt3, Hmcn1, Rptn, Adamts12, Entpd1, Adam28, Col4a4, Col4a3, Wnt10B, Col4a2, Ptprz1, Fbn1, Nid1, Mmp2, Mmp13, Mmp15, Col4a6, Col19a1, Lama3, Vegfa, Tgfbr3, Epyc, Fbln1,	Synthesis and regulation of extracellular matrix proteins
Nog, Tgfbr3, Hif1A, Edn1, Ppp3r1, Zeb2, Tgfbr1, Tgfbr2, Smad2, Smad5, Smad7, Fgf9, Fgfr1, Fgfr2, Egfr, Tgfa, Pdgfra, Itga5, Itga11, Itgb8, Shh, Twist, Snail, Slug, Mmp2, Mmp13, Mmp15, Pip4k2c, Pip5k3, Fndc3a, Fndc5, Fndc1,	Epithelial to mesenchymal transformation
Serinc3, Stk17b, Sirt1, Plagl2, Bcl2l2, Atg7, Dpf2, Bdnf, TAX1BP1, Mitf, Nod2, Sgms1, D430028G21Rik, Bag4, Topors, Tgfbr1, Glrx2, Hif1a, Bcl2l11, Pak7, Egln3, Txndc1, Ihpk2, Trp53inp1, Siah1a, LHX4, Acvr1c, Stk4, AATK, Cdk5r1, Pdcd10, Vegfa, Mnt, Igf1r, Pten, Cadm1, Il8rb, Mcl1, E2f1, Igf1, BFAR, Api5, Camk1 day, Tnfrsf21, Ercc6, Atg12, Ptprc, ATP7A, PRKCA, DBH, Bag2, Col4a3, CASP8AP2, Pdia3, Birc6, Nras, Cdkn1a, Pdcd1lg2	Apoptosis
Tgfbr3, Tgfbr1, Tgfbr2, Smad2, Nr5a2, Pkd2, Txndc10, Clcn3, Txndc1, Pdia6, Adrb2, Pde3b, Stat3, Adrb3, Vegfa, Gpr12, Epas1, Atp2c1, Igf1, Dmd, Aqp4, Tpp1, Ercc6, Edn1, PTHLH, Ptprc, 4930506M07Rik, DBH, ATP7A, Glrx2, PRKCA, Hif1a, Pdia3, Lilrb3, KCNMA1, Socs6, G6pc, Slc39a5, P2rx4,	Epithelial differentiation
Ing2, Ank3, Fgd4, Trappc4, Sh2b1, Neurog2, Sertad2, Ppp6c, Robo2, Robo1, Socs7, Pak4, Arhgef18, Vasp, Dpysl5, IGFBP5, Nedd9, Sfn, Bdnf, Sgms1, Stmn1, Dcx, NFASC, Ndrg3, SEMA3A, Emp1, Prkci, Sema5a, Epha4, Ihpk2, Epb4.1l2, Lhx4, Cxcr4, Abi2, Prrxl1, Rdx, Ptprz1, Stk4, Cdk5r1, Vegfa, Creb1, Epha7, Wasf1, Brms1l, Ulk1, Rnf6, Tsg101, Gas7, Wasl, Mecp2, SLITRK6, Jub, Derl2, Esr1, Ntrk2, ATP7A, Socs6, Lims1, Limk1, Etv1, Barhl2, A730098P11Rik, Tgfbr1, Tns3, Fut8, Camk2n1, Zeb2, Pten, Podxl, Cntn2, Gja1, Cbll1, Itga11, Cspg4, Ccl25, IL16, Dbh, Prkca, Hif1a, Lama3, Amot, Pitx2,	Migration
Pkd2, Sema5a, Frs2, Cxcr4, Acvr1c, Hoxa4, MIB1, Zeb2, Vegfa, Otx2, Pbx3, Bmpr2, ACVR2A, Meox2, Tgfbr2, Hoxd13, Edn1, Mll1, Rnf111, Tgfbr1, Emx2, Pitx2, Mllt3, Sf3b1,Timp2, Fgd4, Kalrn, Fgd5, SLA2, Iqsec2, Rgl1, Adrb2, Net1, Adrb3, Pten, Socs7, Arhgef18, Tiam2, Spred1, Zdhhc17, Atp2c1, Tbc1d15, Nlk, Epgn, Igf1, Sos2, Sos1, Cnksr2, Rabgap1l, Rasa2, Ereg, 4921505C17Rik, Nod2, Zfp91, Nog, SPRY2, Cited2, D430028G21Rik, Itsn2, Ptprc, AKT2, PRKCA, Hif1a, Socs6, Nras, RAPGEF6, ARHGEF3, Fgf9,	Regulation of signal transduction
Rab10, UBTF, Neurog2, Hic2, Sirt1, Zfp583, Plagl2, En2, E2f5, Npas3, Zeb2, Pbx3, Znfx1, Ankib1, Cnbp, Zfp78, Fbxl10, ZBTB39, FLI1, Mitf, FBXL19, Cited2, Sfpi1, Zkscan1, Mllt10, Ankrd49, LHX4, Asb3, Sox17, Jazf1, Zbtb26, Stat3, Slc2a12, Klf12, Brms1l, MLLT6, Bach1, Rfxank, Rnf6, Rab8b, Hivep1, Chaf1a, Ankrd6, Mll1, Maff, Esr1, Gtf2h2, Phf3, Zfp9, Ncoa3, A730098P11Rik, Tle4, Ing2, CREBL1, Zfp40, Lin28, Bzw1, HDAC2, Hoxa4, Smarcb1, Cnot6, PPARA, Zfp354b, Epas1, Lhx6, Narg1, Hlf, Klf6, Zfp619, Mycn, ZBTB6, TAF8, Zfp287, Nlk, Ankrd57, Zfp810, Ets1, CTCF, Hmga2, Tbl1xr1, Arid4b, Zfp800, Zbtb7a, Jmjd1a, Mapk14, Pitx2, Lin28b, Sp2, Lhx8, Mier1, Sall4, AHRR, Crem, Zfp238, Hif1an, Egr2, Creb3l1, PPP1R12B, Tanc2, Mnt, Zfpm2, Meox2, Npas2, Igf1, Wasl, Mecp2, Dach2, Ikbkap, Sirt4, Rbbp7, Txnip, CASP8AP2, Gata3, Zhx2, Etv1, Mllt3, Zfp606, Nr5a2, Cebpz, Rb1, Zfp367, BC066107, 9230110K08Rik, Suhw4, Myst2, SUZ12, EG434179, Dpf2, Irf9, Zbtb5, Mga, Phf8, Irf2bp2, Ercc8, Smad7, Zfp248, Myt1l, Dnajb6, Pou6f1, Pbx1, Ccnt2, Chd9, Lama3, Emx2, BC043476, Elk3, Zbtb45, C330011K17Rik, Lhx9, SLA2, Rsrc1, Adrb2, Ezh1, E2f1, Nfat5, Nkx6-3, ATF1, Lrrfip1, Cand1, Runx1t1, Zfp148, Zfp456, Ankfy1, Sertad2, Nr6a1, Mxd1, Tal1, St18, Hmgb1, Rps6ka5, Pygo2, Zbtb44, Clock, Zfp422-rs1, Cnot7, Hif1a, Tshz3, Ankrd52, Prrxl1, CREBZF, Creb1, TGIF2, Otx2, Mdfic, Zfp655, GATAD2B, Tsg101, Hoxd13, 2410018C20Rik, Zfp110, Zfp91, Tfam, HIVEP2, Foxb1, Barhl2, RBAK, Trpv6	Regulation of transcription, DNA-dependent

[a] Differential expression of the miRNAs (targeting these genes) during orofacial development (GD-12–GD-14) has been verified by TaqMan QRTPCR (see Table 5).

[b] Target analysis of the miRNAs (targeting these genes) was performed using the miRDB (http://mirdb.org/miRDB/), as described in the Methods section.

[c] Enriched gene ontology biological processes (GO BP) for the putative target genes, as predicted by the DAVID software, as described in the Methods section.

[d] For the full list of biological processes associated with the target genes, including *p* value and fold enrichment, see Supplementary Data File 1.

Table 8

Partial List of Target Genes (of Differentially Expressed miRNAs Belonging to Clusters 4, 6 and 5) Known or Likely to Be Associated with Biological (Cellular) Processes Indispensable for Mammalian Palatogenesis

(Cluster #) miRNA name	Target Genes[a,b]	Cellular Process[c,d]
(Cluster 4) *MIR-342-3P: HSA-MIR-342-3P; MMU-MIR-342-3P; RNO-MIR-342-3P; MIR-342: ADDITIONAL SPECIES*	*PAK4, Id4, Cyclin T2, Stk4, Map3k9, Pdgfra, Wnt3, E2F3*	Cell Proliferation and/ or Growth Factor Signaling
(Cluster 6) *MIR-494: HSA-MIR-494; MMU-MIR-494; RNO-MIR-494*	*Fgfr2, Rb1cc1, Pitx2, Gli3, Zic2, Myct1, Fzd2, Igf2bp1, Ccnyl1, Cyclin D2, Cdk12, Api5, Bmpr1a or Alk3, Frs2, Creb1, Crem, Cks1 or Cks1b, Mapk2k6*	
(Cluster 6) *MIR-188-3P: HSA-MIR-188-3P; MMU-MIR-188-3P; RNO-MIR-188-3P*	*Stk3, Mnt, BCL2-like 2 or Bcl-w, Fzd5, E2F6, Ccnyl1, Rap1b*	
(Cluster 6) MIR-212: HSA-MIR-212; MMU-MIR-212; RNO-MIR-212	*Mycbp2, Pnn (Pinin), Sgk3, Btc (betacellulin), Foxp2, Rras2, Bmp2k, Sox5, p300, Nras, Lhx9, Daam1, Erbin, Mapk1 (ERK2), Cyclin G1, TGFβRIII, Bmpr1b or Alk6, FGF10, Rap2b, Gdf5*	
(Cluster 6) *MIR-376B: MMU-MIR-376B; RNO-MIR-376B*	*Lin9, Lefty2, Caprin1, Dlx5, Msx2, Hipk1, Skp2, FGF5, Endothelin-1, Wnt3a*	
(Cluster 5) *MIR-322-MIR-424: MMU-MIR-322; RNO-MIR-322; (MMU-MIR-424); (RNO-MIR-424)*	*N4bp, Col5a1, Onecut2, Cd2ap, Dclk1, Nf1, Tbx22, Mfn2, Lats2, Dmtf1, Col24a, Fermt2, Ectodysplasin-A, Protocadherin 9, Col12a1, Traf3, Mixl1, Sox6, Raf1, Sestrin-1, Ski, Atf6, Cdx2, Siah1a, Rad9, Hoxa10, Dll1*	Apoptosis, Cell adhesion, Extracellular matrix reorganization, Cellular differentiation
(Cluster 5) *MIR-106B: HSA-MIR-106B; MMU-MIR-106B; RNO-MIR-106B*	*Smad7, Tnfrsf21, Txnip, Mfn2, Lama3, Rgma, Rgmb, Pcdhac2, Col4a4, Col4a2, Col19a1, Sema7a, Jak1, pcdhac1, pcdha6, pcdha1, pcdha2, pcdha4, pcdha3, pcdha11, pcdha5, pcdha7, pcdha10, pcdha12, pcdha9, pcdhb4, pcdha8, pcdhb17, pcdh7, Pdcd1lg2, Hif1a, Limk1, TGFβRII, Btg3, Wfs1, Mll1, Bambi, Caspase-12, E2F1, N-myc, Epha7, Timp2, Stat3, Lhx6, Itgb8, p38Mapk or Mapk14, Cdkn1a (P21), Rb1, Creb1, Rbl2 or p130*	
(Cluster 5) *MIR-652: HSA-MIR-652; MMU-MIR-652; RNO-MIR-652*	*Isl1, Frem2*	
(Cluster 5) *MIR-181A: HSA-MIR-181A; MMU-MIR-181A; RNO-MIR-181A*	*Mll1, IL1α, Pten, Ddr2, Nr6a1, Runx1, pcdhac2, pcdhb3, pcdha3, pcdha2, pcdha4, pcdha10, pcdha11, pcdha6, pcdha7, pcdha5, pcdha8, pcdha9, pcdha12, pcdha8, pcdhb17, pcdh18, Gata6, Ccar1, Onecut2, Sox6, Osteoprotegerin (Tnfrsf11b), Itga2, Itgam, Timp3, Cdon, TGFβRI (Alk5), TNF (TNFa), Trail or Tnfsf10, Wasl, Pax9, Pawr*	
(Cluster 5) *MIR-96: HSA-MIR-96; MMU-MIR-96; RNO-MIR-96*	*p75 or Tnfrsf1b, Cd164, Galnt7, Sh3kbp1, Glypican-3, Dock1, Sox6, Ezrin, mTOR, Desmocollin 1, Ext1, Ogt, Map2k1 or Mek1, Cdh20, Mll1, Smad7, Meox2, Zeb1, Celsr1, p120 or Ctnnd1, Plod2*	

[a] Target analysis of the miRNAs (targeting these genes) was performed using the miRDB [(http://mirdb.org/miRDB/)], as described in the Methods section.

[b] Biological processes for the putative target genes were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).