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Methylated MicroRNA Genes of the Developing Murine Palate

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Abstract

Environmental factors contribute to the etiology of cleft palate (CP). Environmental factors can also affect gene expression *via* alterations in DNA methylation suggesting a possible mechanism for the induction of CP. Identification of genes methylated during development of the secondary palate provides the basis for examination of the means by which environmental factors may adversely influence palatal ontogeny. We previously characterized the methylome of the developing murine secondary palate focusing primarily on protein-encoding genes. We now extend this study to include methylated microRNA (miRNA) genes. A total of 42 miRNA genes were found to be stably methylated in developing murine palatal tissue. Twenty eight of these were localized within host genes. Gene methylation was confirmed by pyrosequencing of selected miRNA genes. Integration of methylated miRNA gene and expression datasets identified 62 miRNAs, 69% of which were non-expressed. For a majority of genes (83%), upstream CpG islands (CGIs) were highly methylated suggesting down-regulation of CGI-associated promoters. DAVID and IPA analyses indicated that both expressed and non-expressed miRNAs target identical signaling pathways and biological processes associated with palatogenesis. Furthermore, these analyses also identified novel signaling pathways whose roles in palatogenesis remain to be elucidated. In summary, we identify methylated miRNA genes in the developing murine secondary palate, correlate miRNA gene methylation with expression of their cognate miRNA transcripts, and identify pathways and biological processes potentially mediated by these miRNAs.

Keywords

CpG methylation; methylated regions of interest; mirtrons; palatal ontogeny; pyrosequencing; sister microRNA pairs

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

INTRODUCTION

MicroRNAs (miRNAs) act as ‘regulatory buffers’ that adjust gene expression profiles by overcoming perturbations in gene expression [1, 2]. They play critical roles in regulating mRNA expression during development [3, 4]. Aberrant miRNA expression can, thus, lead to adverse developmental outcomes [5]. miRNAs are ~22 nucleotide (nt) long non-coding RNAs that repress gene expression. Gene silencing is achieved *via* a 6–8 nt ‘seed’ sequence, located at the 5’ end of the molecule, that base pairs with the 3’ untranslated region (UTR) or coding region of target mRNAs resulting in translational inhibition or mRNA degradation [6]. miRNAs that target mRNA coding regions typically promote translational inhibition whereas those that target the 3’UTR facilitate mRNA degradation [7]. The short seed sequence confers miRNAs with their unique ability to target a number of different mRNAs, a feature that also allows mRNAs to be targeted by multiple miRNAs. This redundancy in miRNA action likely explains the lack of overt phenotypes when ablating miRNA function [8].

The morphogenesis of the secondary palate is a complex developmental process that occurs between gestational days (GDs) 12–14 in mice. The secondary palate originates as paired outgrowths (palatal processes) from the oral aspect of the maxillary prominence. In mammals, these outgrowths initially reside lateral to the tongue, and then reorient to a position above the dorsum of the tongue where they fuse with each other, the primary palate anteriorly, and the nasal septum anterodorsally. This fusion occurs between the homologous medial edge epithelia of each palatal process and involves apoptosis, cell migration and/or epithelial mesenchymal transition (EMT) [9–14]. Aberrant development of the secondary palate can result in cleft palate (CP), a defect that manifests in ~2650 babies born in the US each year [15]. Genes that play key roles in palate development predominantly encode members of key signal transduction pathways, such as the Wnt-, TGF β -, PDGF-, FGF- and Shh-signaling systems [10, 16–20]. However, the contribution of miRNAs to secondary palate development has only recently begun to emerge.

The first miRNA to be implicated in palatogenesis was *dre-miR-140* which disrupts PDGF-signaling during zebrafish palate development [21, 22]. A SNP in the cognate human miRNA gene (*hsa-miR-140*) has been identified as a risk factor for non-syndromic CP [23]. *mmu-miR-200b*, expressed in the MEE during palate development, has been found to regulate the expression of *Zeb1* and *Zeb2*, genes considered essential for palatal epithelial fusion [24]. This miRNA has also been implicated in suppressing the effects of TGF β -mediated regulators such as Smad2 and Snail during palatogenesis [25]. Studies using mouse palatal mesenchymal cells identified the *miR-17-92* cluster as an inhibitor of TGF β 1 induced cell proliferation and collagen synthesis, required for ECM formation [26] - specifically, *miR-17* and *miR-20a* targeted *TGFBR2* and, *miR-18a* targeted *Smad2* and *Smad4*. Of relevance, mutation of this cluster has been reported to cause cleft lip and palate (CL/P) by modulating the expression of *Tbx1*, *Tbx3* and *Fgf10*, factors considered essential for midfacial development [27]. Barritt *et al.* [28] identified complete palatal clefts when *Dicer1* was ablated in the *Pax2-Cre/Dicer1* conditional knockout mice. In this model, knockout of *Dicer1* did not affect early events in palatogenesis, such as cranial neural crest (CNC) migration to the first pharyngeal arch or the formation of palatal shelves, but fusion

and mineralization of the palatal shelves were severely compromised. The failure of palatogenesis to proceed was attributed to decreased proliferation and increased apoptosis of CNC-derived mesenchymal cells within the palatal processes proper [28]. Studies from our laboratory have identified ~70 miRNAs that are expressed on each of GDs 12, 13, and 14 (the critical period for murine palato-genesis), with ~60 being common to all three days of gestation [29]. These miRNAs were predicted to affect TGF β -, BMP-, Wnt-, retinoic acid-, JAK/Stat-, VEGF-, PI3K/AKT-, and calcium signaling pathways [29], virtually all of which have been implicated in contributing to proper palatal ontogeny. Overall, these observations emphasize that the differential expression of miRNAs promotes crosstalk among various signaling cascades to effect differentiation and morphogenetic programs of the developing secondary palate.

There is, however, considerable dearth of knowledge on how these miRNAs are regulated during palatogenesis. Specifically, the effects of epigenetic mechanisms such as DNA methylation on miRNA expression have not been documented. We recently characterized the developmental methylome of the murine secondary palate focusing only on protein-encoding genes [30]. Here, we extend these studies and identify methylated miRNA genes in the developing secondary palate, correlate miRNA gene methylation with expression of their cognate miRNA transcripts, and identify pathways and biological processes potentially mediated by these miRNAs.

MATERIALS AND METHODS

Animals

Mice (ICR strain; Harlan, Indianapolis, IN) were used as per approved IACUC protocols and were housed in a climate-controlled room with a 12-hr alternating dark-light cycle. Mice were mated overnight and females were checked for vaginal plugs the following morning (GD0). On GD12, GD13 and GD14, pregnant dams were euthanized by CO₂ asphyxiation and embryos collected in ice-cold calcium/magnesium-free PBS, and immediately processed.

Mouse Secondary Palate and Genomic DNA Isolation

Mouse secondary palates and genomic DNA (gDNA) were isolated as previously described [30]. For each GD (GD12–14), 3 different litters (representing 3 biological replicates) were used for isolation of gDNA. gDNA was isolated from 20 – 40 mg of pooled tissues using the QIAamp DNA mini kit (Qiagen, MD, USA).

Methylation Microarray Experiments

Experimental details, unless otherwise stated, are described in Seelan *et al.* [30]. Briefly, sonicated gDNA was subjected to Selective Enrichment of Methylated DNA (SEMD) using the MethylCollector kit (Active Motif, Carlsbad, CA); the resultant DNA was amplified using a Whole Genome Amplification kit (Sigma; St. Louis, MO) and used as microarray probes. As a control, sonicated gDNA not subjected to SEMD (i.e., input DNA) was similarly processed. Nine microarrays (mouse 2.1M Deluxe Promoter Arrays; Roche NimbleGen, Inc., Madison, WI) representing 3 biological replicates on each of GDs 12, 13

and 14, were probed with either Cy5-labeled SEMD DNA or Cy3-labeled input control DNA. The NimbleGen 2.1M mouse promoter array contains 2.1 million oligonucleotide probes spanning 10 kb of all annotated promoters, all known CpG islands (CGIs), and all miRNA promoters. Signal intensity data, extracted from the scanned images of each array using NimbleScan (NimbleGen), was utilized for analysis.

Determination of Methylated Regions of Interest (MRIs)

Data pre-processing, smoothing of reporter intensities, locating candidate methylated regions of interest (MRIs), relating MRIs to genomic features and determining differentially methylated regions (DMRs) are described in detail in Seelan *et al.* [30]. MRIs were defined as containing six or more contiguous methylated probes within a 900 bp window. The requirement of six contiguous probes avoids identifying methylated regions supported by only a few reporters, and the window width was slightly less than the average fragment size to prevent calling disconnected methylated regions. DMRs between GD12, 13, and 14 were determined using a statistical test based on a linear mixed-effects (LME) model [31] (for details, see Seelan *et al.* [30]). Since no statistically significant DMRs were identified, this was not pursued further in the present study and focus was instead placed on the MRIs. Mouse genome annotation was obtained using Build 37 from the Ensembl database (mm9, July 2007), including Ensembl gene identifiers, MGI (mouse genome informatics) symbols, and descriptions of all genes annotated for the mm9 mouse assembly.

Relating MRIs to miRNA Genes

MRIs were related to miRNA genes using the Bioconductor package *Ringo* [32]. Mouse miRNA locations corresponding to mouse genome mm9 were downloaded using Galaxy (<http://usegalaxy.org/>) [33] in BED format. An MRI was regarded as related to a miRNA gene if its center position was located less than 500 bases upstream of the gene's start coordinate, or between a gene's start and end coordinates. miRNA genes associated with MRIs were determined separately for each GD, and the union of the miRNA genes used to characterize the set of methylated miRNA genes during murine palate development. The union was selected since the methylation profiles did not differ substantially across GD 12–14 (see Seelan *et al.* [30]). The presence of CGIs within a 1–3 kb region spanning an MRI was detected using the program CpG Island Searcher [34] using default parameters. CGI length was set at 200 bp.

Integration of MiRNA Gene Methylation with MiRNA Expression

Analysis of miRNA expression levels during murine palate development was done previously by Mukhopadhyay *et al.* [29] using two-color miRXplore™ miRNA expression arrays (Miltenyi Biotech GmbH, Germany). Details of RNA extraction, microarray preprocessing, normalization, and statistical analysis are given in that reference. Since the MRIs associated with miRNA genes did not exhibit significant differential methylation, we focused on identifying miRNA genes that were 'expressed' vs. 'non-expressed' rather than differentially expressed. To accomplish this, the raw microarray data was used to identify miRNAs that had median intensity values in the red (experimental) channel that were greater than 1.3 times the red background ('expressed') vs. those that were not ('non-expressed').

These two lists of miRNAs were subsequently analyzed for interesting biological pathways, as detailed below.

Pathway Analysis

Determination of the biological relevance of the methylated miRNA genes was done using multiple approaches. First, targets of miRNAs categorized as ‘expressed’ and ‘non-expressed’ were determined using the intersection of targets in miRBase [35] (currently available as MicroCosm Targets at <http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), via the Bioconductor package *microRNA* [36], and TargetScan [37], via the Bioconductor package *target-scan.Mm.eg.db* [38]. These predicted targets of methylated miRNAs were assessed for enrichment in GO categories and biological pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG) [39] and Panther [40] using DAVID [41, 42]). Secondly, expressed and non-expressed miRNAs were analyzed using the Ingenuity Pathway Analysis for involvement in biological pathways and functional categories. Highly predicted and/or experimentally verified gene targets of miRNAs were assessed and their involvement in various canonical cascades was analyzed using Ingenuity Pathway Analysis program (IPA; Ingenuity Systems, Redwood City, CA). Within IPA, miRNA gene targets were mined using the following databases –TargetScan: http://www.targetscan.org/mmu_50/; Tarbase [43]; miRecords [44].

Determination of CpG Methylation Levels by Pyrosequencing

Methylation levels of CpG residues found within five genomic regions encompassing miRNA genes (*mir-93*, *mir-127*, *mir-142*, *mir-376c*, and *mir-541*) were determined by pyrosequencing. Genomic DNA from GD12, GD13 and GD14 secondary palates was bisulfite-modified using the EpiTect Bisulfite kit (Qiagen). Primers for the amplification of bisulfite-modified DNA were custom designed using the PyroMark Assay Design 2.0 software (Qiagen). The primers and the genomic regions subjected to pyrosequencing are depicted in Table S1. Samples were PCR amplified using the PyroMark PCR kit (Qiagen), according to the manufacturer’s instructions, and pyrosequenced in a PyroMark Q24 instrument (Qiagen) from, at least, 2 independently amplified PCR templates.

RESULTS

Identification of Methylated MiRNA Genes During Secondary Palate Development

NimbleGen 2.1M Deluxe Promoter microarrays (see Methods) were used to identify methylated regions of interest (MRIs) in tissue derived from the developing murine secondary palate on gestational days (GD) 12–14 [30]. MRIs were defined as regions containing *six* or more contiguous methylated probes within a 900 bp window (see Methods). A total of 42 miRNA encoding genes exhibiting stable methylation on all 3 GDs were associated with MRIs (center position located between 500 bases upstream of TSS and end coordinate of gene) (Table 1, Fig. S1).

Validation of the Methylated MiRNA Genes of the Developing Secondary Palate

To validate the methylated miRNA genes identified from analysis of promoter arrays, the methylation status of CpG residues found within 5 miRNA genes (*mir-93*, *mir-127*, *mir-142*,

mir-376c, and *mir-541*; Fig. S1) was determined by pyrosequencing. These included 3 from the list of 42 methylated genes exhibiting high, intermediate or low methylation and 2 potentially unmethylated genes (*mir-376c* and *mir-541*; Fig. S1); moreover, their gene sequences were permissive to the amplification of the region of interest from bisulfite-modified DNA. For location of CpG residues, see ‘*Determination of CpG methylation levels by pyrosequencing*’ in Materials and Methods. Data derived by pyrosequencing (Table 2) were consistent with those observed by promoter array analysis. The methylation of a miRNA gene was arbitrarily considered to be low, intermediate or high based on the methylation of probes for all three replicates on all 3 GDs (see Fig. S1).

Comparison of the methylation status of miRNA genes observed by promoter array analysis with average CpG methylation levels (computed for all CpGs on all 3 GDs) of the targeted DNA region in the cognate genes is as follows: (1) *mir-93* (low - intermediate methylation): $66 \pm 4\%$; (2) *mir-127* (high methylation): $96 \pm 5\%$; (3) *mir-142* (intermediate methylation): $68 \pm 12\%$; (4) *mir-376c* (low methylation): $50 \pm 2\%$; and, (5) *mir-541* (low - intermediate methylation): $83 \pm 12\%$ - in this case, the high methylation value was contributed by a single highly methylated CpG site (Table 2). Overall, none of the CpG residues exhibited significant differential methylation from GD12 to GD14 (p-value < 0.05 and 10% change in CpG methylation levels). Thus, all CpG sites analyzed maintained stable methylation levels from GD12 to GD14.

Integration of MiRNA Methylation with MiRNA Expression

The expression of mature miRNAs in the developing murine secondary palate during each of GDs 12–14 was previously reported by our laboratory [29]. This expression dataset permitted comparisons between the 42 stably methylated miRNA genes and the expression of their cognate mature miRNAs. Out of a possible 84 miRNAs (each miRNA gene can potentially encode a pair of mature miRNAs derived from each arm of the pre-miRNA stem-loop structure), probes for 62 miRNAs were identified in the expression array. Using a signal (median red (R) or experimental channel intensity level) to noise (median red background (Rb) intensity level) ratio of 1.3, miRNAs were categorized as *non-expressed* ($R/Rb \leq 1.3$) or *expressed* ($R/Rb > 1.3$). The value of 1.3 was selected based on our previous analysis of similar data [46]. By this criterion, 43 miRNAs (~69%) were considered *non-expressed* and the rest, *expressed* (19, ~31%) (Figs. S2 and S3, respectively; Table 3). Though a majority of the miRNAs were considered non-expressed, this percentage was not significantly different from the percentage of miRNAs originating from *unmethylated* genes that were considered not expressed analyzed by the same criteria ($407/514 = 79\%$ non-expressed vs. $107/514 = 21\%$ expressed, $p = 0.14$).

Pathway Analysis

Both non-expressed and expressed miRNAs were subjected to Pathway analyses (Tables S2a and S2b, respectively). Target analysis (as described in *Pathway Analysis* under Materials and Methods) resulted in 1665 and 1046 putative targets for non-expressed and expressed miRNAs, respectively. Pathway and gene ontology (GO) analyses by DAVID [47] were conducted separately (Tables 4A and 4B, respectively). Power for gene-set analysis is closely tied with the number of genes annotated to the biological database, and a

lower significance threshold (DAVID modified Fisher exact test p-value (EASE score) < 0.01) was used for pathway databases relative to GO databases ($p < 1e-05$). See Tables S2a and S2b for DAVID settings used for each analysis.

Comparison of both categories of miRNAs indicated that they regulate identical as well as distinct pathways and signaling processes commonly associated with palatogenesis. Mechanisms involving Wnt, Notch, TGF β , and MAPK, and pathways associated with focal adhesion, actin cytoskeleton regulation and adherens junction, to name a few, were enriched in both categories of miRNAs (Table 4A). However, other pathways pertinent to palatogenesis were found to be enriched only in non-expressed miRNAs (*e.g.*, FGF signaling and ECM receptor interaction) or expressed miRNAs (*e.g.*, PDGF signaling).

An examination of the top 20 GO terms for both non-expressed and expressed miRNAs (Tables S2a and S2b, respectively) highlighted neuronal, morphogenetic and transcriptional properties which likely reflect the neural crest origin of these cells and the underlying biochemical processes associated with secondary palate morphogenesis. Overall, GO terms common to both expressed and non-expressed miRNAs include several terms applicable to palatogenesis - examples include 'cell morphogenesis involved in differentiation' and 'tissue Morphogenesis', to name a few (Table 4B). Likewise, GO terms enriched only for non-expressed or expressed miRNAs also highlighted palate morphogenesis (Table 4B).

Experimentally validated targets of both sets of miRNAs, as determined in miRTarBase (Release 4.5) [49] are provided in Tables S2a and S2b for both non-expressed and expressed miRNAs. The majority of the non-expressed miRNAs (36/43, 84%) did not have verified targets in the database, while the remaining 7 miRNAs had only one to two verified targets with the exception of miR-758-3p, which had 239 weakly supported functional miRNA-target interactions via a single sequencing study [50]. In contrast 11 of the 19 (58%) expressed miRNAs had one to four experimentally verified targets, determined by a variety of methods including luciferase reporter assays and 5'RACE.

Ingenuity Pathway Analysis (IPA) program also revealed that both categories of miRNAs target thousands of genes that encode growth factors/morphogens, signaling mediators, transcription factors, and extracellular matrix proteins, among others (Tables 5 and S3). Intriguingly, many of these gene targets are targeted by multiple miRNAs (Table S3). All of these gene targets are either highly predicted and/or experimentally verified and they are directly or indirectly linked to an array of signaling pathways and/or cellular processes documented to be indispensable for palate development (*e.g.*, signaling mechanisms associated with apoptosis, actin cytoskeleton, adherens junction, BMP, TGF β , FGF, Wnt/ β -catenin, Notch, PDGF, Sonic hedgehog, cyclins and cell cycle regulation, and regulation of EMT pathway, among others). It is apparent that the various signaling cascades are fine-tuned by the action of several miRNAs belonging to the two categories (expressed and non-expressed) studied in this report.

DISCUSSION

Few studies have attempted to link alterations in epigenetic processes such as DNA methylation with the etiology of facial clefting [51]. By focusing primarily on methylated genes encoding proteins, we previously characterized the developmental methylome of the murine secondary palate [30]. Because of their critical roles in palate development (as elaborated in the *Introduction*), we now extend this study to include methylated genes encoding miRNAs. Methylated regions of interest (MRIs) in the genome (operationally defined as regions harboring 6 contiguous methylated probes within 900 bp) were first identified using the algorithm developed in the Bioconductor package [52] *Ringo* [32], and then associated with annotated genomic elements using *BiomaRt* [53]. A MRI was related to a miRNA locus only if its center position was located < 500 bases upstream of the gene's start site or between a gene's start and end sites. Potentially methylated regions not satisfying our criteria for a MRI were, in the present study, considered to be unmethylated. Thus, more subtle effects of methylation (lower levels of methylation or locations further upstream) may have been missed in the present analysis.

A total of 42 methylated miRNA genes, stably methylated on all 3 GDs, were identified (Table 1). Five genes were selected for independent validation by pyrosequencing of CpG residues. These selected miRNA genes included 3 methylated and 2 unmethylated genes that exhibited low, intermediate, or high methylation levels as determined by methylation array profiling (Fig. S1). Analysis revealed consistency with the methylation array data (Table 2). Furthermore, the results from these arrays were previously validated by pyrosequencing of CpG residues found within the MRIs of 8 protein-encoding genes [30]. Some discordance between the array results and pyrosequencing is to be expected, as pyrosequencing measures methylation levels of individual CpG sites, located adjacent to each other, whereas array results reflect aggregate signals from probes, spaced 100 bp apart and harboring a varied number of CpG sites methylated to different levels. For example, while the array results indicated that the *mir-541* gene is localized in a region displaying low to intermediate methylation on all 3 GDs (Fig. S1), pyrosequencing revealed that one of the CpG residues within this region is highly methylated (CpG2; > 98%) during the developmental time points analyzed (Table 2). Differentially methylated regions (DMRs) were not identified in this study. This could be due to: lack of sufficient power in our promoter array analysis; paucity of DMRs during the period of palatogenesis analyzed; or, to the technique used to identify MRIs in the genome [30] which may have been biased against regions of low CpG density. Supporting this notion is the fact that tissue-specific DMRs are more likely to reside in CpG-poor or non-promoter intergenic regions [54,55].

The 42 methylated miRNA genes we identified include both intergenic and intragenic miRNAs. Interestingly, 24 of the methylated miRNA genes are located within a ~180 kb region on chromosome 12. The intragenic miRNAs are embedded within non-coding RNA (ncRNA) as well as protein-encoding host genes, spanning introns and exons, in sense and antisense orientations (Table 6). These miRNAs harbor the potential to regulate host gene expression either antagonistically or synergistically [56–58]. The 28 intragenic miRNAs, some of which occur in clusters, reside within 22 host genes. GO analysis (MGI database) and published evidence suggest that several of these miRNAs regulate developmental

processes known to be associated with palatal ontogeny (Table 6 and references therein), such as, apoptosis, epigenetic regulation, response to growth factors, and signaling mechanisms involving insulin receptor, protein kinase B, Wnt and BMP. Four of the intragenic miRNA genes (*mir-127*, *mir-370*, *mir-675* and *mir-770*) are located within imprinted loci, regions that exhibit parent-of-origin methylation patterns. Of particular relevance is the observation that the host gene for *miR-675*, *H19*, has been implicated in the etiology of CP in *TGFβ3^{-/-}* mice [59]. In their study, using RNA-Seq, Ozturk *et al.* [59] observed that *H19* was one of few genes to exhibit altered expression in the palates of *TGFβ3^{-/-}* mice, compared to those of *TGFβ3^{+/-}*. The list of methylated miRNA genes also includes at least two mirtrons - *miR-702* and *miR-877*. Unlike many canonically processed pri-miRNAs which are cleaved by Drosha, mirtrons are generated by intron splicing, bypassing Drosha cleavage [60].

The effects of miRNA gene methylation on miRNA expression in the developing palate were determined by integrating the datasets of methylated miRNA genes with that of a previously characterized miRNA transcriptome [29]. Based on probes common to both the promoter methylation arrays (2.1 M Deluxe Promoter Arrays, Roche NimbleGen, Madison, WI, USA) and miRNA expression arrays (miRX-plore microarrays, Miltenyi Biotec, Bergisch Gladbach, Germany), a total of 62 miRNAs were identified. A predominant number of these miRNAs (43; 69%) were non-expressed while the remaining 19 (31%) were expressed. However, this percentage of non-expressed miRNAs did not differ statistically from the percentage of miRNAs that were not expressed from unmethylated miRNA genes. Nonetheless, for a majority of the methylated miRNA genes (35/42; 83%), the MRI peaks perfectly overlapped CpG islands (CGIs) (Fig. S1, black bars) which were typically located upstream of genes (within 1400 bp of the 5' end of genes) or overlapped them. It is thus likely that the observed suppression of the expression of a majority of miRNAs may be due to methylation of CGI-associated promoters. However, a majority of miRNA genes considered as expressed by our criteria also had methylated CGIs upstream of the gene, indicating that in these cases, mechanisms other than promoter methylation contribute to their expression.

Since the methylated miRNA genes in the developing secondary palate were found to be associated with both expressed and non-expressed miRNAs, it was of interest to determine the pathways and biological processes mediated by these two categories of miRNAs. DAVID (Tables 4A and 4B) and IPA pathway (Table 5) analyses indicated that both categories of miRNAs target identical as well as distinct signaling pathways and biological processes, relevant to secondary palate morphogenesis (see RESULTS). The association of some of these pathways (notably, actin nucleation by ARP-WASP complex, 14-3-3-mediated signaling, p53 signaling, mTOR signaling, and neurotrophin signaling) with palatogenesis is less well known, or in some cases, novel, and warrants further exploration. Interestingly, a recent study has observed that p53 overexpression during early embryogenesis could contribute to a variety of developmental defects, including craniofacial defects, such as CP [61]. DAVID analyses also indicated that there are several pathways and processes that are uniquely targeted by each category of miRNA (Tables 4A and 4B). The data presented suggest the hypothesis that both expressed and non-expressed miRNAs

associated with the methylated miRNA genes may act in concert to fine-tune cellular processes underlying palatogenesis. Two examples in 'Wnt signaling' and 'Tgf β signaling' may illustrate this point. Bioinformatic analyses suggest that Wnt signaling through Wnts 2b, 9a and 10b likely occurs during palatogenesis, as miRNAs targeting their cognate transcripts are *not expressed* (see 'Wnt signaling' targets under 'Pathways' - Table S2a). In contrast, APC is required for abrogating Wnt signaling by phosphorylating and degrading β -catenin [66]. In our analysis, the *Apc* transcript is a target of *expressed* miRNAs (see 'Wnt signaling' targets under 'Pathways' - Table S2b), suggesting inhibition of *Apc* expression. Thus, these two classes of miRNAs presumably collaborate to augment signaling by the Wnt molecules. In the second example, our data suggest the putative expression of *Bmp7* and the activation of its downstream effector *Smad5* in the developing palate, as miRNAs targeting the cognate transcripts are *not expressed* (see 'Tgf β signaling' targets under 'Pathways' - Table S2a); this signaling may be further reinforced by the *expression* of miRNAs targeting the transcripts of *Smads 6* and *7* (see 'Tgf β signaling' targets under 'Pathways' - Table S2b). Smads 6 and 7 are inhibitory Smads that can suppress BMP signaling by inhibiting Smad5 phosphorylation [67]. Another area where these two classes of miRNAs can act together pertains to the number of seed sites present in a transcript and the number of miRNAs bound to these sites at any given time. A combinatorial effect of miRNAs binding to a transcript can potentiate or weaken the severity of inhibition [68]. One may thus assume that the binding of one miRNA (expressed) but not another (non-expressed) to the same transcript could influence the strength of translational inhibition or mRNA degradation. Indeed, there are several examples in our analysis of expressed and non-expressed miRNAs targeting the same transcript, including that of *Apc* and *Smads 6* and *7*, discussed above. While the aforementioned examples represent possible mechanisms by which the two classes of miRNAs can act in conjunction, they remain highly speculative until proven experimentally. Because a miRNA gene can potentially encode mature miRNAs from both strands (arms) of the pre-miRNA hairpin structure (i.e., miRNA/miRNA* or miRNA-3p/miRNA-5p), it was of interest to determine how these sister miRNA pairs [69] were expressed during the development of the secondary palate. In most cases, both members of the pair appeared to be identically expressed (or not expressed). For instance, sister miRNA pairs were expressed for two genes (*mir-411* and *mir-434*; Fig. S3) and not expressed for eleven genes (*mir-142*, -323, -328, -369, -338, -433, -490, -666, -675, -770, and -877; Fig. S2). However, selective preference for one strand was observed for seven genes (*mir-93*, -106b, -218, -300, -376a, -409, and -431).

Because the synthesis of mature miRNAs and strand selection occur post-transcriptionally in the cytoplasm, these events are independent of DNA methylation. Thus the biogenesis and maturation of miRNAs encoded by the 42 methylated miRNA genes are quite complex. While, a majority of miRNAs are processed by canonical Drosha-dependent cleavage pathways, a few (e.g., mirtrons) utilize Drosha-independent, intron splicing mechanisms. For miRNA genes residing within host genes, transcription may occur from a unique promoter, or the promoter may be co-opted from the host gene. Moreover, different mechanisms may be utilized for generating mature miRNAs residing in exons versus those in introns of host genes [56]. Thus, a proper assessment of the role of DNA methylation in

the expression of the different miRNAs requires a more detailed evaluation. The present data provides a good starting point for addressing this issue.

CONCLUSION

In this study, we have identified several miRNA genes that are stably methylated throughout the period of secondary palate development. Genetically programmed changes in DNA methylation occur during the normal course of an organism's development - these include processes, such as X-chromosome inactivation, genomic imprinting, retrotransposon silencing, establishment of tissue specificity, cell differentiation, and gestational aging [70–72]. Gene methylation levels can also be affected by genomic mutations that affect imprinting centers or the enzymes participating in DNA methylation/demethylation reactions [73–75]. The contribution of environmental factors to gene methylation changes is well known. Exposures to xenobiotics, famine, stress, or nutritional deprivation during a mother's pregnancy are known to lead to diseases in later life *via* epigenetic changes [70, 71, 76]. It has been documented that folate deprivation in the maternal diet or exposure to tobacco smoke, alcohol or pollutants during pregnancy can disrupt signaling pathways that underlie orofacial development [10]. It would be interesting to examine what impact, if any, the demethylation of the aforementioned miRNA genes has on normal palatogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

BMP	Bone morphogenetic protein
CGI	CpG Island
CL/P	Cleft lip with cleft palate
CP	Cleft palate
DMR	Differentially methylated region
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK/MAPK	Extracellular signal-regulated kinase/Mi-togen-activated protein kinase
FGF	Fibroblast growth factor
GD	Gestational day

gDNA	Genomic DNA
IPA	Ingenuity pathway analysis
IGF-1	Insulin-like growth factor-1
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
MEE	Medial edge epithelium
miRNA	MicroRNA
MRI	Methylated region of interest
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
Shh	Sonic hedgehog
TGFβ	Transforming growth factor β
TSS	Transcriptional start site
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

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Table 1

Methylated miRNA genes and transcripts in murine secondary palate development. The 42 methylated miRNA genes stably methylated on all 3 days of mouse secondary palate development (GD 12-14) are listed along with their mature miRNA transcripts (where known). Alternate or prior nomenclature for the miRNAs is provided within parenthesis. Note that the list does not indicate the expression of miRNAs in the developing secondary palate. Information obtained from miRBase [45].

miRNA genes	miRNAs – 5p arm	miRNAs – 3p arm
<i>mmu-mir-93</i>	<i>miR-93-5p</i> (miR-93)	<i>miR-93-3p</i> (miR-93*)
<i>mmu-mir-106b</i>	<i>miR-106b-5p</i> (miR-106b)	<i>miR-106b-3p</i> (miR-106b*)
<i>mmu-mir-107</i>	<i>miR-107-5p</i> (miR-107*)	<i>miR-107-3p</i> (miR-107)
<i>mmu-mir-127</i>	<i>miR-127-5p</i> (miR-127*)	<i>miR-127-3p</i> (miR-127)
<i>mmu-mir-142</i>	<i>miR-142-5p</i>	<i>miR-142-3p</i>
<i>mmu-mir-143</i>	<i>miR-143-5p</i> (miR-143*)	<i>miR-143-3p</i> (miR-143)
<i>mmu-mir-218-1</i>	<i>miR-218-5p</i> (miR-218)	<i>miR-218-1-3p</i> (miR-218-1*)
<i>mmu-mir-218-2</i>	<i>miR-218-5p</i> (miR-218)	<i>miR-218-2-3p</i> (miR-218-2*)
<i>mmu-mir-299a</i>	<i>miR-299a-5p</i> (miR-299/miR-299*/miR-299-5p)	<i>miR-299a-3p</i> (miR-299/miR-299-3p)
<i>mmu-mir-300</i>	<i>miR-300-5p</i> (miR-300*)	<i>miR-300-3p</i> (miR-300)
<i>mmu-mir-323</i>	<i>miR-323-5p</i>	<i>miR-323-3p</i> (miR-323)
<i>mmu-mir-328</i>	<i>miR-328-5p</i> (miR-328*)	<i>miR-328-3p</i> (miR-328)
<i>mmu-mir-329</i>	<i>miR-329-5p</i> (miR-329*)	<i>miR-329-3p</i> (miR-329)
<i>mmu-mir-338</i>	<i>miR-338-5p</i>	<i>miR-338-3p</i> (miR-338)
<i>mmu-mir-369</i>	<i>miR-369-5p</i>	<i>miR-369-3p</i>
<i>mmu-mir-370</i>	<i>miR-370-5p</i> (miR-370*)	<i>miR-370-3p</i> (miR-370)
<i>mmu-mir-376a</i>	<i>miR-376a-5p</i> (miR-376a*)	<i>miR-376a-3p</i> (miR-376a)
<i>mmu-mir-379</i>	<i>miR-379-5p</i> (miR-379)	<i>miR-379-3p</i> (miR-379*)
<i>mmu-mir-381</i>	<i>miR-381-5p</i> (miR-381*)	<i>miR-381-3p</i> (miR-381)
<i>mmu-mir-409</i>	<i>miR-409-5p</i>	<i>miR-409-3p</i> (miR-409)
<i>mmu-mir-410</i>	<i>miR-410-5p</i> (miR-410*)	<i>miR-410-3p</i> (miR-410)
<i>mmu-mir-411</i>	<i>miR-411-5p</i> (miR-411)	<i>miR-411-3p</i> (miR-411/miR-411*)
<i>mmu-mir-412</i>	<i>miR-412-5p</i>	<i>miR-412-3p</i> (miR-412)
<i>mmu-mir-431</i>	<i>miR-431-5p</i> (miR-431)	<i>miR-431-3p</i> (miR-431*)
<i>mmu-mir-433</i>	<i>miR-433-5p</i> (miR-433*)	<i>miR-433-3p</i> (miR-433)
<i>mmu-mir-434</i>	<i>miR-434-5p</i>	<i>miR-434-3p</i>
<i>mmu-mir-487b</i>	<i>miR-487b-5p</i> (miR-487b*)	<i>miR-487b-3p</i> (miR-487b)
<i>mmu-mir-490</i>	<i>miR-490-5p</i>	<i>miR-490-3p</i> (miR-490)
<i>mmu-mir-539</i>	<i>miR-539-5p</i> (miR-539)	<i>miR-539-3p</i>
<i>mmu-mir-543</i>	<i>miR-543-5p</i> (miR-543*)	<i>miR-543-3p</i> (miR-543)
<i>mmu-mir-598</i>	<i>miR-598-5p</i> (miR-598*)	<i>miR-598-3p</i> (miR-598)
<i>mmu-mir-666</i>	<i>miR-666-5p</i> (miR-666)	<i>miR-666-3p</i>
<i>mmu-mir-675</i>	<i>miR-675-5p</i>	<i>miR-675-3p</i>

miRNA genes	miRNAs – 5p arm	miRNAs – 3p arm
<i>mmu-mir-678</i>	<i>miR-678</i>	
<i>mmu-mir-686</i>	<i>miR-686</i>	
<i>mmu-mir-692-2</i>	<i>miR-692</i>	
<i>mmu-mir-702</i>	<i>miR-702-5p</i>	<i>miR-702-3p</i> (<i>miR-702</i>)
<i>mmu-mir-758</i>	<i>miR-758-5p</i> (<i>miR-758*</i>)	<i>miR-758-3p</i> (<i>miR-758</i>)
<i>mmu-mir-770</i>	<i>miR-770-5p</i>	<i>miR-770-3p</i>
<i>mmu-mir-877</i>	<i>miR-877-5p</i> (<i>miR-877</i>)	<i>miR-877-3p</i> (<i>miR-877*</i>)
<i>mmu-mir-1197</i>	<i>miR-1197-5p</i> (<i>miR-1197*</i>)	<i>miR-1197-3p</i> (<i>miR-1197</i>)
<i>mmu-mir-1892</i>	<i>miR-1892</i>	

Methylation levels of CpG residues in selected miRNAs. Five genes were selected for validation by pyrosequencing analysis of CpG residues in a PyroMark Q24 (Qiagen) instrument. CpGs to be analyzed were determined by the PyroMark Design 2.0 software (Qiagen). Mean methylation levels for each CpG residue were deduced from at least 3 pyrosequencing runs using two independently amplified PCR templates. Statistical significance was computed using a Student's *t*-test for changes in methylation levels between different GDs. No significant changes (*p*<0.05; 10% change) were observed.

Table 2

Methylation levels (%; Mean ± S.D.)				Methylation Promoter array ^a	
		GD12	GD13	GD14	
<i>mir-93</i>	CpG1	66.8 ± 12.8	68.5 ± 9.0	73.0 ± 4.2	Intermediate
	CpG2	63.3 ± 7.4	61.5 ± 6.9	64.5 ± 0.7	
<i>mir-127</i>	CpG1	88.3 ± 3.8	90.0 ± 12.7	89.0 ± 7.1	High
	CpG2	99.3 ± 0.6	100.0 ± 0	99.5 ± 0.7	
	CpG3	100.0 ± 0	100.0 ± 0	100.0 ± 0	
<i>mir-142</i>	CpG1	76.7 ± 2.3	79.5 ± 2.1	78.5 ± 0.7	Intermediate
	CpG2	57.3 ± 4.9	59.5 ± 3.5	55.5 ± 0.7	
<i>mir-376c</i>	CpG1	50.5 ± 2.1	51.0 ± 5.7	48.0 ± 1.4	Low
<i>mir-541</i>	CpG1	83.5 ± 3.5	74.0 ± 11.3	71.3 ± 14.7	Low-Intermediate
	CpG2	100.0 ± 0	97.5 ± 3.5	98.7 ± 2.3	
	CpG3	78.0 ± 0	71.0 ± 7.1	76.0 ± 8.3	

^a based on the methylation of probes for all 3 replicates on all three GDs (see Fig. S1)

Table 3

miRNAs Encoded By Methylated MiRNA Genes Of The Developing Murine Secondary Palate.

Non-Expressed		
<i>miR-93-3p</i>	<i>miR-106b-3p</i>	<i>miR-127-5p</i>
<i>miR-142-3p</i>	<i>miR-142-5p</i>	<i>miR-218-1-3p</i>
<i>miR-218-2-3p</i>	<i>miR-299-3p</i>	<i>miR-300-5p</i>
<i>miR-323-3p</i>	<i>miR-323-5p</i>	<i>miR-328-3p</i>
<i>miR-328-5p</i>	<i>miR-338-3p</i>	<i>miR-338-5p</i>
<i>miR-369-3p</i>	<i>miR-369-5p</i>	<i>miR-370-3p</i>
<i>miR-376a-5p</i>	<i>miR-409-5p</i>	<i>miR-412-3p</i>
<i>miR-431-3p</i>	<i>miR-433-3p</i>	<i>miR-433-5p</i>
<i>miR-487b-3p</i>	<i>miR-490-3p</i>	<i>miR-490-5p</i>
<i>miR-539-5p</i>	<i>miR-543-3p</i>	<i>miR-598-3p</i>
<i>miR-666-5p</i>	<i>miR-666-3p</i>	<i>miR-675-3p</i>
<i>miR-675-5p</i>	<i>miR-678</i>	<i>miR-686</i>
<i>miR-692</i>	<i>miR-702-3p</i>	<i>miR-758-3p</i>
<i>miR-770-3p</i>	<i>miR-770-5p</i>	<i>miR-877-3p</i>
<i>miR-877-5p</i>		
Expressed		
<i>miR-93-5p</i>	<i>miR-106b-5p</i>	<i>miR-107-3p</i>
<i>miR-127-3p</i>	<i>miR-143-3p</i>	<i>miR-218-5p</i>
<i>miR-299-5p</i>	<i>miR-300-3p</i>	<i>miR-329-3p</i>
<i>miR-376a-3p</i>	<i>miR-379-5p</i>	<i>miR-381-3p</i>
<i>miR-409-3p</i>	<i>miR-410-3p</i>	<i>miR-411-3p</i>
<i>miR-411-5p</i>	<i>miR-431-5p</i>	<i>miR-434-3p</i>
<i>miR-434-5p</i>		

Table 4A

DAVID analysis - Putative pathways targeted by miRNAs encoded by methylated miRNA genes during secondary palate development. Pathways relevant to secondary palate development and enriched for targets of the two indicated miRNA groups (non-expressed and expressed), as identified using gene-set enrichment analysis in DAVID [47]. Fold enrichment was determined by the ratio of the number of putative target genes associated with each term divided by the number of putative target genes to the total number of genes associated with each term divided by the total number of mouse genes. The number of miRNAs and the number of putative targets associated with each pathway are also given. Significance levels are indicated by asterisks (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$), with p-values adjusted for multiple comparisons using the Benjamini-Hochberg [48] method.

Non-expressed	Expressed					
	Fold			Fold		
Pathways	Enrichment	#Genes	#miRNAs	Enrichment	#Genes	#miRNAs
Wnt signaling	2.17**	26	31	2.35*	17	14
Regulation of actin cytoskeleton	2.52***	44	38	2.09*	22	16
Adherens junction	2.29*	14	25	3.80**	14	16
Insulin signaling	2.07**	23	31	1.94	13	13
mTOR signaling	3.00**	13	21	2.68	7	10
Neurotrophin signaling	2.30**	24	35	2.06	13	11
Notch signaling	2.74*	14	26	2.48	6	7
Focal adhesion	2.83***	45	37	1.77	17	14
MAPK signaling	2.21***	47	39	1.87*	24	16
TGF- β signaling	3.00***	21	30	2.61	11	12
ErbB signaling	3.00***	21	31	2.14	9	11
FGF signaling	1.79*	27	33	--	--	--
Dorso-ventral axis formation	3.96*	7	15	--	--	--
ECM-receptor interaction	2.55**	17	31	--	--	--
Tight junction	2.30**	25	31	--	--	--
VEGF signaling	2.01*	18	29	--	--	--
EGF receptor signaling	1.71*	28	36	--	--	--
Integrin signaling	1.63*	43	38	--	--	--
PDGF signaling	--	--	--	1.59	22	14

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Non-expressed	Expressed					
	Fold			Fold		
Pathways	Enrichment	#Genes	#miRNAs	Enrichment	#Genes	#miRNAs
Ubiquitin mediated proteolysis	--	--	--	2.58*	17	15
Reelin signaling	--	--	--	5.97	3	8

DAVID analysis - Gene ontology analysis of miRNAs encoded by methylated miRNA genes during secondary palate development. Gene ontology (GO) terms relevant to secondary palate development and enriched for targets of the two indicated miRNA groups (non-expressed and expressed), as identified using gene-set enrichment analysis in DAVID [47]. For details, see legend to Table 4A.

Table 4B

	Non-expressed			Expressed		
	Enrichment	#Genes	#miRNAs	Enrichment	#Genes	#miRNAs
GO TERMS						
Transcription repressor activity	2.91***	52	40	2.78***	31	17
Cell morphogenesis involved in differentiation	2.78***	50	40	2.77***	31	18
Morphogenesis of a branching structure	2.55***	27	36	3.34***	22	15
Tube development	2.46***	55	40	2.80***	39	16
Regulation of transcription from RNA Pol II promoter	2.35***	123	41	2.37***	77	18
Cell morphogenesis	2.33***	61	41	2.27***	37	18
Transcription activator activity	2.28***	50	38	2.55***	35	17
Tissue morphogenesis	2.18***	44	39	2.55***	32	18
Protein serine/ threonine kinase activity	1.88***	67	41	2.16***	48	17
Cartilage development	2.87***	19	28	--	--	--
Embryonic organ development	2.25***	46	36	--	--	--
Morphogenesis of an epithelium	2.25***	33	38	--	--	--
Epithelium development	2.00***	46	40	--	--	--
Regulation of Rho protein signal transduction	2.74**	20	23	--	--	--
Regulation of Ras protein signal transduction	2.73***	42	36	--	--	--
Cell leading edge	2.59***	22	35	--	--	--
Small GTPase regulator activity	2.33***	44	39	--	--	--
Cell migration	2.26***	46	39	--	--	--
Chromatin modification	2.20***	44	37	--	--	--
Cell motion	2.18***	68	39	--	--	--
Muscle organ development	2.14**	32	37	--	--	--

GO TERMS	Non-expressed			Expressed		
	Enrichment	#Genes	#miRNAs	Enrichment	#Genes	#miRNAs
Enzyme linked receptor protein signaling pathway	2.12***	49	40	--	--	--
Pattern specification process	2.20***	53	39	--	--	--
Cell motility	1.95***	47	39	--	--	--
Cell junction	1.80***	64	39	--	--	--
Cytoskeletal protein binding	1.68**	59	40	2.05***	45	18
Protein kinase activity	1.72***	85	41	2.01***	62	17
Phosphate metabolic process	1.61***	118	41	1.90***	87	18
DNA binding	1.74***	262	41	1.73***	163	18
Regulation of transcription, DNA dependent	1.64***	204	41	1.68***	18	

Table 5

Ingenuity pathway analysis – putative signaling pathways targeted by methylated miRNA genes during secondary palate development.

Signaling pathway	Methylated miRNA genes	
	Non-Expressed	Expressed
<i>Number of experimentally verified and/or highly predicted targets*</i>		
14-3-3-mediated	33	36
Actin cytoskeleton	57	71
Actin nucleation by ARP-WASP Complex	11	20
Apoptosis	23	29
BMP	23	30
Cyclins and cell cycle regulation	18	32
DNA methylation & transcriptional repression	5	8
Epithelial adherens junction	36	55
ERK/MAPK	56	67
FGF	26	37
IGF-1	28	40
Integrin	54	60
JAK/STAT	20	27
Mouse ES cell pluripotency	35	51
Notch	9	16
P38 MAPK	30	31
P53	30	33
PDGF	23	26
Regulation of EMT	58	86
Sonic hedgehog	5	10
STAT3	29	40
TGF β	25	44
Wnt/ β -catenin	54	88

* miRNA target analysis was performed using the Ingenuity Pathway Analysis Program (IPA; Ingenuity Systems, Redwood City, CA).

Table 6

Methylated MicroRNA Genes And Their Host Genes.¹

miRNA gene	Location	Host Gene	Host Gene Name	Gene ontology/ Gene product attributes
<i>mmu-mir-93</i> <i>mmu-mir-106b</i>	Intron (s)	<i>Mcm7</i>	Minichromosome maintenance deficient 7 (<i>S.cerevisiae</i>)	cellular response to inorganic substance; regulation of viral genome replication; Wnt signaling pathway; <i>in utero</i> embryonic development; negative regulation of apoptosis; negative regulation of B cell apoptosis.
	Intron (s)	“	“	Wnt signaling pathway; cellular response to inorganic substance; <i>in utero</i> embryonic development; negative regulation of apoptosis; negative regulation of B cell apoptosis.
<i>mmu-mir-107</i>	Intron (s)	<i>Pank 1</i>	Pantothenate kinase 1	cellular response to amino acid stimulus; cellular response to lipopolysaccharide; response to oxygen levels.
<i>mmu-mir-127</i> <i>mmu-mir-431</i> <i>mmu-mir-433</i> <i>mmu-mir-434</i>	Exon (as)	<i>Rtl1</i>	Retrotransposon like 1	epigenetic regulation of gene expression; response to oxygen levels.
	“	“	“	
	“	“	“	
	“	“	“	cellular hyperosmotic salinity response
<i>mmu-mir-142</i>	Intron (s)	<i>A430104N18Rik</i>	(lincRNA)	cellular response to estrogen stimulus
<i>mmu-mir-143</i>	Intron (s)	<i>Mir143hg</i>	Mir143 and Mir145 host gene (ncRNA)	cellular response to estrogen stimulus; embryo implantation; negative regulation of insulin receptor signaling; negative regulation of protein kinase B signaling; regulation of glucose metabolism; aorta smooth muscle tissue morphogenesis; cardiac vascular smooth muscle cell differentiation.
<i>mmu-mir-218-1</i>	Intron (s)	<i>Slit2</i>	Slit homolog 2	regulation of endothelial cell migration; retina vasculature development; Roundabout signaling pathway;
<i>mmu-mir-218-2</i>	Intron (s)	<i>Slit3</i>	Slit homolog 3	regulation of endothelial cell migration; retina vasculature development; Roundabout signaling pathway;
<i>mmu-mir-328</i>	Exon/ Intron (as)	<i>Elmo3</i>	Engulfment and cell motility 3	
<i>mmu-mir-338</i>	Intron (s)	<i>Aatk</i>	Apoptosis associated tyrosine kinase	cellular response to growth factor stimulus; myelination; negative regulation of osteoblast differentiation.
<i>mmu-mir-369</i> <i>mmu-mir-410</i> <i>mmu-mir-412</i> <i>mmu-mir-409</i>	Exon/ Intron (s)	<i>Mirg</i>	MicroRNA containing gene (ncRNA)	cellular response to glucose stimulus
	Exon (s)	“	“	cellular response to glucose stimulus;- regulation of insulin secretion.
	Exon/ Intron (s)	“	“	
	Intron (s)	“	“	
<i>mmu-mir-370</i>	Intron (s)	<i>Rian</i>	RNA imprinted and accumulated in nucleus (ncRNA)	
<i>mmu-mir-490</i>	Intron (s)	<i>Chrm2</i>	Cholinergic receptor, muscarinic 2	
<i>mmu-mir-598</i>	Intron (s)	<i>Xkr6</i>	X Kell blood group precursor related family member 6 homolog	

miRNA gene	Location	Host Gene	Host Gene Name	Gene ontology/ Gene product attributes
<i>mmu-mir-675</i>	Exon (s)	<i>H19</i>	H19, imprinted maternally expressed transcript (ncRNA)	Regulation of BMP pathway in skeletal muscle [62]; placental growth [63].
<i>mmu-mir-678</i>	Exon (s)	<i>Prmt2</i>	Protein arginine N-methyltransferase 2	
<i>mmu-mir-686</i>	Exon (s)	<i>Psmb5</i>	Proteasome subunit, β type 5	
<i>mmu-mir-692-2</i>	Intron (s) Exon (s)	<i>Grik3</i> <i>Ftl2</i>	Glutamate receptor, ionotropic, kainite 3 Ferritin light chain 2	
<i>mmu-mir-702</i>	Exon/ Intron (s)	<i>Plod3</i>	Procollagen-lysine, 2-oxo glutaraldehyde 5 dioxygenase 3	Regulation of apoptosis [64]; cell cycle regulation [65].
<i>mmu-mir-770</i>	Exon (s)	<i>Meg3</i>	Maternally expressed imprinted gene (ncRNA)	
<i>mmu-mir-877</i>	Intron (s)	<i>Abcf1</i>	ATP binding cassette, subfamily F, member 1	
<i>mmu-mir-1892</i>	Exon (s)	<i>Sptssa</i>	Serine palmitoyltransferase, small subunit a	

¹ information obtained from miRBase, MGI and NCBI databases or cited references; miRNA genes that occur in clusters are boxed together. (s), sense; (as), antisense;