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Defining the rhesus macaque placental miRNAome: conservation of expression of placental miRNA clusters between the macaque and human

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Abstract

Objective—Expression of microRNAs (miRNAs) in the human placenta is dynamic across gestation, with expression of miRNAs belonging to the C14MC, C19MC and miR-371-3 clusters. Specifically, miRNAs within the C19MC cluster are exclusively expressed in primates with predominant expression in the placenta. Non-human primates can be utilized to study developmental processes of placentation *in vivo* that cannot be assessed in the human placenta, however, miRNA expression has not been defined in the macaque placenta. Our objective was to profile miRNAs in the macaque placenta, hypothesizing that expression is conserved between the macaque and human placenta.

Methods—Total RNA from first trimester and term macaque placentas (n=4 per group) was analyzed through RNA-sequencing and validated by quantitative real-time PCR (qRT-PCR).

Results—A total of 607 pre-miRNAs previously annotated in the macaque reference database (miRBase21) were detected, and 166 miRNAs were differentially expressed between first trimester and term placentas. A total of 457 unannotated sequences were detected and deemed candidate novel miRNAs by miRDeep2 software. Differential expression was confirmed for six of nine miRNAs evaluated by qRT-PCR. Comparative analysis demonstrated expression of several miRNA orthologs of human pregnancy-associated miRNA clusters in the macaque placenta.

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Conclusions—Profiling placental miRNAs of the macaque revealed conserved expression of a number of miRNAs within the C14MC, C19MC and miR-371-3 clusters between the human and macaque. These results establish non-human primates as a model for human placentation and miRNA biology, with the prediction of their functional significance in placental development and function.

Keywords

macaque; placenta; miRNA; gene expression

INTRODUCTION

Nonhuman primates are excellent models for studying the biology of human pregnancy. Although interstitial trophoblast invasion is limited within the decidua compared to human placentation [1, 2], similarities to human placentation in the villous structure, endovascular trophoblast migration and spiral artery remodeling as well as a longer duration of gestation and maturity of young are strengths of the macaque model in comparison with other animal models. Additionally, the immune cell population, endocrine profile, and placental MHC expression are similar between nonhuman primate and human placentas [1, 3].

The central events of implantation and pregnancy are thought to be highly regulated by molecular cues, including the expression of microRNAs (miRNAs). miRNAs are small (18–25 nucleotides) non-coding RNAs that post-transcriptionally regulate gene expression by binding to target mRNAs to repress or degrade the transcripts [4, 5], and act to upregulate transcription through direct and indirect mechanisms [5–7]. miRNAs are involved in diverse cellular processes including embryonic development [8, 9], implantation [10] and pregnancy [11–13]. In humans, there are three miRNA clusters (MCs) highly expressed within the placenta, the C14MC, C19MC and miR-371-3 clusters. The C14MC is expressed from the maternally inherited chromosome and includes 52 miRNAs located within the imprinted DLK-DIO3 domain of human chromosome 14 [11, 13, 14]. The C19MC is expressed from the paternally inherited allele within an imprinted region and contains 46 miRNA genes [13, 15, 16]. The miR-371-3 cluster is adjacent to the C19MC and consists of 3 conserved mammalian miRNAs [13] that are highly expressed in embryonic stem cells with roles in cell cycle regulation [17–19]. The miRNAs of these clusters have roles in placental function and associations with placental pathologies such as preeclampsia [11–13, 20–23]. For instance, miR-519d of the C19MC cluster may indirectly alter extravillous trophoblast migration through regulation of target transcripts CXCL6, NR4A2 and FOXL2 [24]. The C19MC is of particular interest as it is exclusively expressed in primates and predominantly in the placenta [16, 25] with high expression in human trophoblasts [26, 27].

The macaque model has been well defined in terms of placental structure and physiology, however, the expression of miRNAs within the macaque placenta has been essentially unexplored. The objective of this study was to profile miRNA expression within the macaque placenta, hypothesizing that miRNA expression is conserved between macaques and humans. Through a miRNA-sequencing (miR-seq) approach, miRNA expression profiles were defined for first trimester and term macaque placentas. These profiles will be

informative for future studies in the macaque model to better understand molecular changes underlying placental developmental processes and pathologies.

MATERIALS AND METHODS

Animals

Rhesus monkeys (*Macaca mulatta*) were from the colony maintained at the Wisconsin National Primate Research Center. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Graduate School Animal Care and Use Committee. A total of 16 placentas were collected from pregnancies terminated in the first trimester (gestation day: 36) or from term clinical cesarean sections (gestation day: ~165).

RNA Extraction

RNA extraction was performed using TRIzol Reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) following protocol recommendations. Total RNA was extracted from 4 first trimester and 4 term placentas and sent to Arraystar Inc. (Rockville, Maryland, USA) to undergo miR-seq.

Library Preparation and miRNA-sequencing

A miR-seq library was prepared for each sample through the following steps: ligation of a 3'-adapter and 5'-adapter, cDNA synthesis, PCR amplification, and extraction and purification of the amplified PCR fragments from a PAGE gel. The sequencing libraries were quantified with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). Each sample was diluted to 8 pM and cluster generation was performed on the Illumina cBot using a TruSeq Rapid SR cluster kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina NextSeq 500 using TruSeq Rapid SBS kits (Illumina). All kit protocols were performed following the manufacturer's instructions.

Raw Data Processing and Differential Expression Analysis

Raw sequences were generated as clean reads by real-time base calling and quality filtering. Reads were trimmed of the 3'-adaptor sequence and reads shorter than 15 nt were discarded. The trimmed reads were then aligned to rhesus macaque pre-miRNA sequences in the miRBase 20 reference database (<http://www.mirbase.org>), using novoalign software for annotation and generation of miRNA read counts, which were used to estimate the expression level of each miRNA. Expression was normalized as transcripts per million total aligned miRNA reads (TPM). Differential expression was calculated by deriving the fold change of the log₂ transformed TPM for each miRNA, and a t-test was performed to determine statistical significance ($P < 0.05$). All known macaque miRNAs of the pregnancy-associated clusters were analyzed for changes in expression level from first trimester to term. A 2-way ANOVA was performed using GraphPad Prism (version 7) software for all miRNAs in the cluster at different stages of gestation where significance was defined as $P < 0.05$.

Expression Validation by qRT-PCR

In addition to the 8 sequenced samples, an additional 8 placentas (4 first trimester and 4 term) were extracted for total RNA as described above. Samples were diluted to 80 ng/ul and a spike-in control, *C. elegans* mature miR-39c, was added to each sample (6.24×10^5 copies per sample). Total RNA was reverse transcribed using a miRNA II RT kit (Qiagen, Germantown, MD) following the manufacturer's recommended protocol, utilizing the HiSpec buffer and 920 ng of sample. The cDNA was then diluted 1:5 with RNase-free water and qRT-PCR reactions were assembled by using a miScript SYBR green PCR kit (Qiagen) combined with a primer specific to the miRNA of interest (see Supplementary File 1 for primer sequences). All reactions were performed in triplicate and a set of no template control reactions were included in parallel for each primer. The qRT-PCR reactions were performed as recommended by the kit protocol and run on a Roche Light Cycler. The spike-in control served as the reference gene for all samples to calculate the C_t . Data were analyzed by performing a t-test on the C_t values for each miRNA and are graphically represented as the fold change in expression \pm the standard error using the 2^{-C_t} method by Livak and Schmittgen [28].

C14MC, C19MC, and miR-371-3 Cluster Analysis

miRNAs belonging to the human pregnancy associated clusters were analyzed for conservation of expression between human and non-human primate placentas and trends in expression across gestational age. If a human miRNA within one of these clusters was not represented in the macaque dataset, the human miRNA sequence was analyzed by BLAST and miRBase to determine sequence complementarity between the known human miRNA and the rhesus genome or annotated miRNA reference sequences. Sequences conserved in each cluster were assessed for expression changes throughout gestation. Statistical analysis was performed using GraphPad Prism software. A 2-way ANOVA with post-hoc Bonferroni Correction for multiple comparisons was applied to the mean log transformed TPM read count for each miRNA in the cluster at each time point and significance was defined as $P < 0.05$.

Novel miRNA Candidate Analysis

Unannotated macaque sequences were analyzed by miRDeep2 software for prediction of novel miRNAs. Briefly, a score of the likelihood that a sequence represents a novel miRNA candidate is assigned based on an algorithm that takes into account the number of reads corresponding to the mature and star sequences and also, the relative and absolute stabilities of the sequence structure [29, 30]. To characterize the predicted novel sequences, a BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed to align the predicted sequences to the macaque genome (assembly Mmul_8.0.1) and then also to the human genome (assembly GRCh38.p7) if a macaque miRNA annotation did not exist. Additionally, the predicted sequence was searched in miRBase to determine sequence alignments between the macaque and human.

RESULTS

miRNA-Sequencing Analysis of the Rhesus Macaque Placenta

The miRNA expression profiles of 4 first trimester and 4 term rhesus macaque placentas were evaluated by miR-seq analysis. Reads were aligned to annotated rhesus pre-miRNAs within miRBase, where the average number of reads aligned to pre-miRNAs were about 9,476,574 and 8,701,971 for first trimester and term placentas, respectively (Supplementary File 2). A total of 1064 sequences were expressed with at least 1 TPM in one sample, where 607 sequences aligned to known rhesus macaque pre-miRNAs and 457 novel miRNA candidate sequences were predicted through miRDeep2 analysis. Non-supervised hierarchical clustering of all expressed miRNAs revealed segregation of individual placentas based on gestational age with distinct expression patterns between early and late gestation (Figure 1A). A larger proportion of miRNAs were more highly expressed by greater than 2-fold change in first trimester versus term placentas, in distinction from the reverse comparison (Figure 1B).

Differential Expression of miRNAs in First and Term Rhesus Macaque Placentas

Across gestation, a total of 166 miRNAs were significantly differentially expressed (> 2 -fold), where 137 and 29 miRNAs were preferentially expressed in first trimester and term placentas, respectively (Table 1). Supplementary file 3 provides information in regards to the predominant miRNA isoforms expressed, mean fold change in expression between groups, the p -value, and false discovery rate (FDR) for each differentially expressed miRNA. Figure 2 shows the cluster analysis of differentially expressed miRNAs in individual samples (top dendrogram), and in addition, differentially expressed miRNAs clustered in terms of their relative expression levels (left dendrogram). Despite the fact that each of these miRNAs were differentially expressed, most miRNAs segregated as low, moderate or high expression clusters that were maintained across both first trimester and term placentas. Additionally, a subset of six miRNAs were highly abundant as well as differentially expressed (Figure 2, bottom of the heat map).

To provide technical validation of the miR-seq differential expression results, expression was evaluated for 9 selected miRNAs by qRT-PCR in sequenced RNA samples ($n=4$) and in additional biological replicates ($n=4$) for each first trimester and term placentas. miRNA selected for qRT-PCR analysis included two novel miRNA candidates (Chr19_17320 and Chr19_16465), two C14MC miRNAs (miR-323 and miR-134), two C19MC miRNAs (miR-519a and miR-518c), two miR-371-3 cluster miRNAs (miR-371-5p and miR-372), and an additional miRNA preferentially expressed in term placentas (miR-139). Differential expression was confirmed for Chr19_17320, miR-372, miR-371-5p, miR-519a, miR-518c and miR-139, whereas expression was not significantly different between first trimester and term placentas for Chr19_16456, miR-323 and miR-134 (Figure 3). These results confirm that human pregnancy-related C19MC and miR-371-3 cluster miRNAs are differentially expressed at different stages of gestation in the macaque placenta.

Conservation of miRNA expression between macaque and human placentas

miRNAs belonging to the human C14MC, C19MC and miR-371-3 placenta-associated clusters were assessed for conservation of expression between the human and macaque placenta. Figure 4 illustrates the annotation of macaque miRNAs representing orthologs of the human placenta-associated clusters, where each gene and species (–5p and/or –3p distinction) is depicted across the chromosome. If a human miRNA was not represented in the macaque dataset, a BLAST of the human pre-miRNA sequence to the macaque genome was performed to determine the existence of a macaque ortholog. The human C14MC is positioned on macaque chromosome 7 and of the 52 miRNAs in the cluster, 45 genes (76 species) were expressed, 4 were not expressed and 3 human miRNAs, mir-1197, mir-300 and mir-655, had no sequence similarities between the macaque and human. Overall, ~88% of all expressed C14MC species had higher expression in first trimester compared to term placentas. A comparison of expression levels across gestation for each miRNA within the cluster by a 2-way ANOVA revealed a significant difference ($P < 0.0001$), suggesting decreased expression of the C14MC through gestation (Figure 4B, Supplementary File 4). The trends in expression for all miRNAs within each cluster are represented in Supplementary File 4.

Conserved expression of the C19MC was observed in the macaque as 32 of 46 genes were expressed, however, several known human miRNAs within this cluster are not annotated in the macaque (Figure 4A). No sequence similarities were observed between the human and macaque for human pre-miRNA sequences mir-515-1, mir-515-2 and mir-520e. BLAST of human C19MC pre-miRNA sequences to the macaque genome revealed only partial alignments to several annotated macaque C19 miRNAs, and thus a specific ortholog or homolog could not be distinguished. Altogether, 49 C19MC species were expressed in our dataset, ~71% were higher in first trimester. As illustrated in Supplementary File 4, the C19MC miRNA species demonstrate a wide range of expression levels in both the first and third trimesters. A comparison of expression levels between early and late gestation for all C19MC miRNAs revealed a significant difference ($P < 0.0001$, 2-way ANOVA with post-hoc Bonferroni Correction) in expression, and supported the apparent trend of higher expression in first trimester placentas. The miR-371-3 cluster had significantly ($P < 0.0001$) different expression levels from first trimester to term.

Characterization of Novel Macaque miRNA Candidate Sequences

A total of 286 sequences had a miRDeep score greater than 5 and an 87% estimated probability that the miRNA sequence was a true positive call. The pre-miRNA candidate sequences were aligned to the rhesus genome and mature sequences were searched in miRBase. Nine novel candidate sequences aligned with 0 mismatches to known human mature miRNAs, 19 with 1 mismatch, 10 with 2 mismatches and 17 with > 2 mismatches (Table 2). Although sequences with partial alignments could not be fully characterized, we propose that novel rhesus sequences with complete alignments to known human miRNAs may be considered macaque miRNA candidates.

DISCUSSION

The expression profile of macaque placental miRNAs was established through a miR-seq approach. Unsupervised clustering of miRNA expression levels in individual placentas revealed distinct profiles for placentas collected in first trimester compared to those collected at term, suggesting that miRNA expression is specific to the stage of pregnancy and consistent across individuals. We predict that study of the macaque placenta miRNAome and their respective target genes will provide a deeper understanding of the molecular basis of physiology and pathophysiology in macaque placental studies.

Profiles of highly expressed placental miRNAs vary across human studies, which may be attributable to gestational age, the placental component analyzed, and/or methods used to assess expression. Luo et al. [31] reported high expression of miR-21, miR-125b and miR-517a in human placentas regardless of gestational age or representative cell types assessed. These miRNAs were also detected in the current study, although miR-148a-3p, miR-26a-5p and miR-516b were the most highly expressed macaque placental miRNAs, suggesting that species-specific differences in miRNA expression may exist. A microarray analysis of human first trimester and term placentas identified 191 differentially expressed genes, of which miRNAs belonging to the miR-17-92, C14MC, C19MC and miR-371-3 clusters were highly expressed in first trimester placenta [32]. miRNAs within these clusters were also detected in first trimester macaque placentas in the present study.

Differential expression of the pregnancy-associated miRNA clusters has been observed in human placentas across gestational age. Luo et al. [31] reported predominant expression of miRNAs from chromosome 19 in whole villous term placenta samples. Similarly, Morales-Prieto et al. [26] reported high expression of C19MC miRNAs in primary term human trophoblasts, whereas members of the C14MC were predominantly expressed in first trimester trophoblasts. The authors suggest that expression of the C14MC decreases and the C19MC increases throughout gestation. In agreement with human studies, ~88 % of the C14MC miRNAs expressed in the macaque placenta decreased from first trimester to term, however, predominant expression of the rhesus C19MC members in term placenta was not observed. This is in concordance with another human study by Gu et al. [32], and differences in C19MC expression patterns could be attributed to the representative placental cell types analyzed.

Genomic sequences of known human and macaque miRNAs were examined in the present study and the results were compared to a previous study conducted by Morales-Prieto et al. [20]. While miRNAs of the miR-371-3 cluster are well conserved across the human and macaque, a subset of the human C14MC and C19MC members were not orthologous in the macaque. Morales Prieto et al. [20] determined that no macaque orthologs exist for hsa-mir-655 of the C14MC or hsa-mir-518a-1, hsa-mir-518a-2 and hsa-mir-520h of the C19MC. In the present study, about 87 % of the human C14MC genes were expressed in the macaque, however, no macaque orthologs could be determined for 3 human C14MC miRNAs, mir-1197, mir-300 and mir-655. In comparison, the homology between the human and macaque C19MC is more complex as 3 human miRNAs have no sequence similarities with the macaque and 11 human miRNAs did not have a macaque ortholog, although a

subset partially aligned to the macaque genome. Additionally, the sequence of macaque mir-517b was more closely matched to human mir-517a or mir-519b, hence there are C19MC sequence differences between humans and macaques that warrant further investigation. It has been demonstrated that macaque pre-miRNA and mature miRNA sequences are more dissimilar to human sequences compared to other non-human primates such as chimpanzee or orangutan [33]. Thus, determining orthologs of human miRNAs in the rhesus macaque requires more careful exploration and ultimately functional testing.

In summary, expression profiling of miRNAs in the macaque placenta revealed conserved expression of miRNAs belonging to pregnancy-associated clusters. Given the importance of rhesus macaques in studies to model human pregnancy, further work is needed to improve the annotation and explore both the putative targets and functional roles of C19MC members in the macaque placenta. As miRNAs serve to regulate gene expression in a variety of tissues including the placenta, a greater understanding of the expression profile of the macaque placental miRNAs will enhance interpretation of the biology at the maternal-fetal interface in response to experimental conditions or pathologies in the macaque animal model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Macaque placenta miRNA expression changes across placental development.
- The human pregnancy-associated miRNA clusters are conserved in the macaque.
- Novel predicted macaque miRNA sequences were identified.

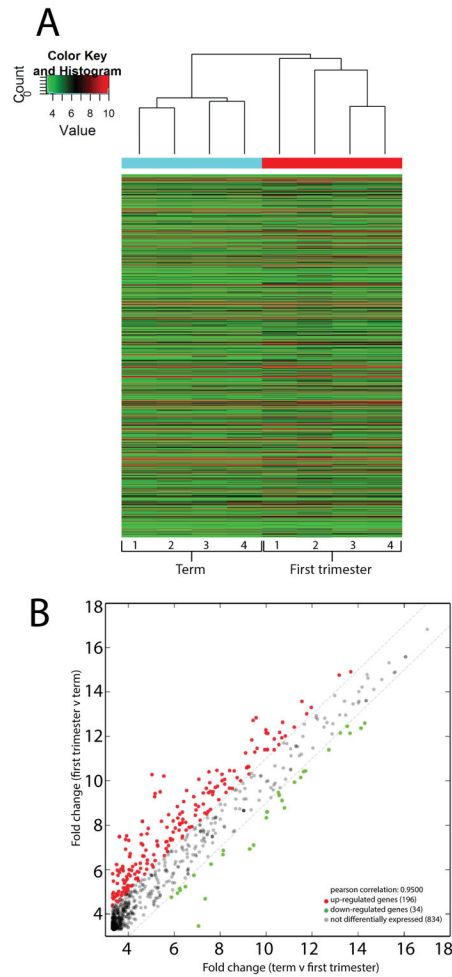


Figure 1. miRNA expression in the macaque placenta. **A.** Unsupervised hierarchical clustering of miRNA expression. Each row represents an expressed miRNA and each column represents a sequenced placenta in which the first trimester placentas (n=4) and the term placentas (n=4) are distinctly clustered. Expression levels increase from green to red. **B.** Fold change in expression is represented for first trimester compared to term placentas on the y-axis and term compared to first trimester on the x-axis to depict expression levels between placentas of differing gestational age. Each miRNA is represented by a point where red are miRNAs highly expressed in first trimester, green are miRNAs highly expressed in term and gray are miRNAs not differentially expressed.

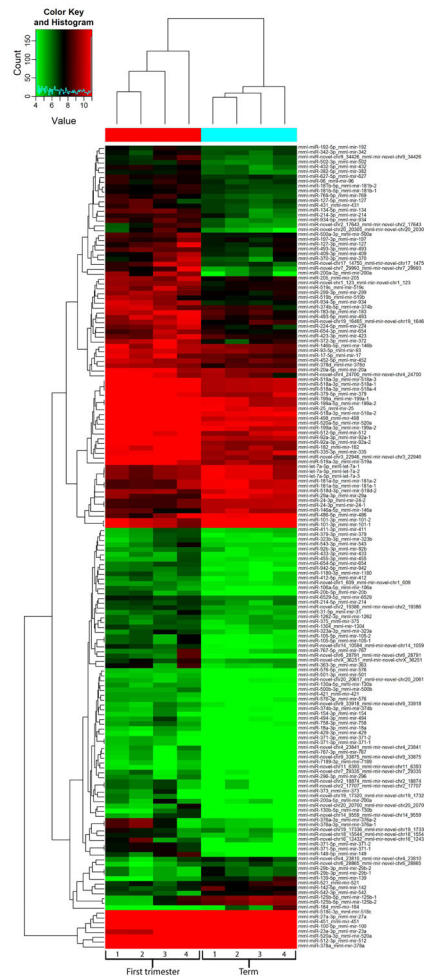
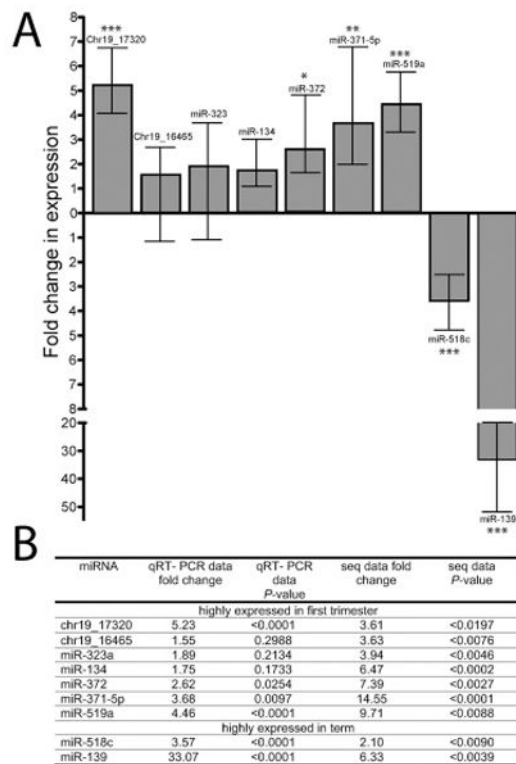
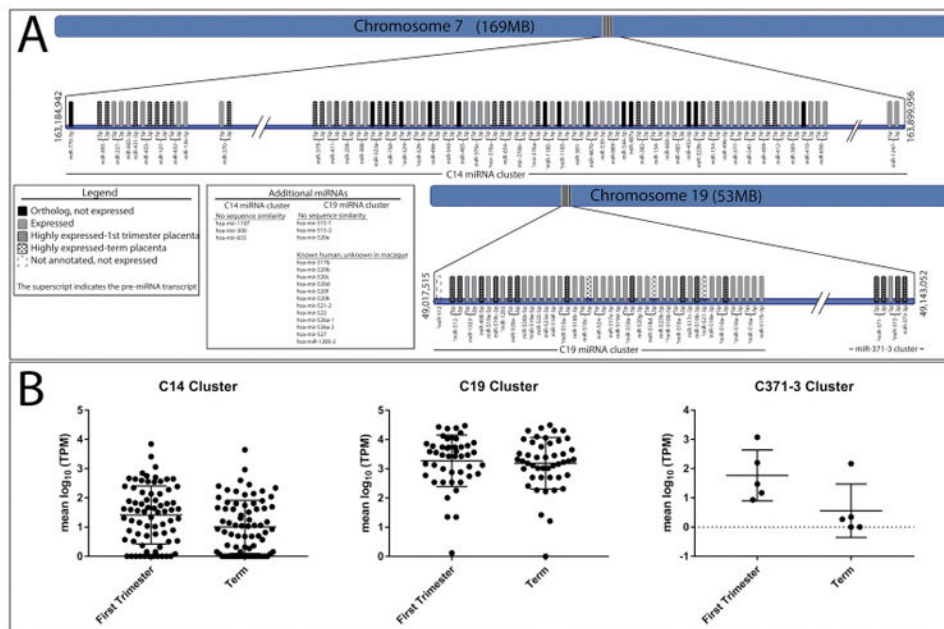


Figure 2. Hierarchical clustering of differentially expressed miRNAs between first trimester and term placentas. The top dendrogram illustrates the relatedness in expression across placentas, whereas the left dendrogram represents clustering of miRNAs with similar expression levels. The highest levels of expression are indicated in red and the lowest level of expression are indicated in green.

**Figure 3.**

Gene expression validation by qRT-PCR. A. Fold change in expression between first trimester and term placentas was assessed for nine selected miRNAs. B. The fold change in expression obtained by qRT-PCR and RNA-sequencing with their corresponding *P*-values. *P*-values are denoted as follows: * *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.0001.

**Figure 4.**

Analysis of the conservation of placenta-associated miRNA clusters between the macaque and human. **A.** Annotated miRNAs within the C14MC, C19MC and miR-371-3 clusters were assessed in macaque placentas. Comparison in expression between the human and macaque is represented by individual bars along the chromosome designated as either an ortholog but not expressed, expressed, highly expressed in first trimester placenta, highly expressed in term placenta, and not annotated or expressed but is an ortholog. Highly expressed refers to those that are significantly, differentially expressed. **B.** Expression of miRNAs within placenta-associated clusters across gestation. The mean log₁₀TPM for each miRNA and the overall mean and SE for all miRNAs is represented for each time point. Significantly different ($P < 0.0001$) expression levels between first trimester and term placentas for the C14MC, C19MC and miR-371-3 cluster are denoted by an asterisk.

Table 1

Differentially expressed miRNAs in macaque placentas.

Provisional ID	Sequence 5'-3'	Homolog
0 mismatches		
Chr1_2078	mml- aaaaguaauuggguuuuugcc hsa- aaaaguaauuggguuuuugcc	*precursor mml-mir- 548j hsa-miR-548d-5p
Chr3_21665	mml- ugucuuacucccucaggcacau hsa- ugucuuacucccucaggcacau hsa- ugucuuacucccucaggcacau	*precursor mml-mir- 550 hsa-miR-550a-3p hsa-miR-550b-2-5p
Chr6_27880	mml- ugugagguuggcauuguugucu hsa- ugugagguuggcauuguugucu	hsa-miR-1294
Chr7_31026	mml- ugugacaauagagaugaacaug hsa- ugugacaauagagaugaacaug	hsa-miR-4504
Chr14_10402	mml- gagagcugagacuagaaagcc hsa- gagagcugagacuagaaagcc	hsa-miR-3160-5p
Chr13_8652	mml- uugcagcugccugggagugacuuc hsa- uugcagcugccugggagugacuuc	hsa-miR-1301-3p
Chr15_11847	mml- agauguccagcccaaaucucg hsa- agauguccagcccaaaucucg	*precursor mml-mir-219-2 hsa-miR-219-5p
Chr16_13207	mml- aucauguaugauacugcaaaaca hsa- aucauguaugauacugcaaaaca	hsa-miR-6516-3p
Chr19_16465	mml- gaagaucucaggcugugac mml- gaagaucucaggcugugac	*precursor mml-miR-517b mml-miR-517b ¹
Chr20_20972	mml- cuggccucucugccuuccgu hsa- cuggccucucugccuuccgu	*precursor mml 20-2162 miRNA hsa-miR-328-3p
1 mismatch		
Chr1_111	mml- ucaucccccucgcccucacaga hsa- ucaucccccucgcccucacag-	hsa-miR-6729-3p
Chr1_2567	mml- cacaugggccaaaacagagaagg mml- cacaugggccaaaacagagaag- hsa- cacaugggccaaaacagagaaga	*precursor mml-miR-942 mml-miR-942-5p hsa-miR-942-5p
Chr1_3220	mml- -cacagcaaguguagacaggcag mml- gcacagcaaguguagacaggca- hsa- gcacagcaaguguagacaggac-	*precursor mml-mir-214 mml-miR-214-5p hsa-miR-214-5p
Chr3_22381	mml- aguugccagggcugccuuuggg hsa- aguugccagggcugccuuuggu	hsa-miR-6501-5p
Chr4_23772	mml- ucagguguggaaaacugaggcagg hsa- ucagguguggaaaacugaggcag-	hsa-miR-3934-5p
Chr7_29354	mml- uggguuuacguugggagaacc hsa- uggguuuacguugggagaacu	hsa-miR-629-5p
Chr7_30131	mml- aucauagaggaaaauccacau mml- aucauagaggaaaauccacgu hsa- aucauagaggaaaauccacgu	*precursor mml-mir-376a mml-miR-376a-3p hsa-miR-376a-3p
Chr7_30497	mml- ccucagggcuguagaacaggacu hsa- ccucagggcuguagaacaggcuc	hsa-miR-1266-5p
Chr9_34605	mml- uuccauugcauauacuucuuu- hsa- uuccauugcauauacuucuuug	hsa-miR-202-5p
Chr9_33862	mml- uccugucuuuccuuguggag- hsa- uccugucuuuccuuguggagc	hsa-miR-5599-3p
Chr10_3642	mml- uggaguccaggaauucgcauuu- hsa- uggaguccaggaauucgcauuuu	hsa-miR-1289

Chr10_3650	mml- aaacaucacugcaagucuaac mml- aaacaucacugcaagucuaa- hsa- aaacaucacugcaagucuaa-	*precursor mml-mir-499 mml-miR-499-5p hsa-miR-499a-5p
Chr12_7679	mml- aaaaaccacaa <u>uu</u> acuuuugc hsa- aaaaaccacaa <u>uu</u> acuuuugcacca hsa- aaaaaccacaa <u>uu</u> acuuuugcacca	*precursor mml-mir-548g hsa-miR-548aa hsa-miR-548t-3p
Chr14_9450	mml- aaaaaccacaa <u>uu</u> acuuuugc hsa- aaaaaccacaa <u>uu</u> acuuuugcacca hsa- aaaaaccacaa <u>uu</u> acuuuugcacca	*precursor mml-mir-548d hsa-miR-548aa hsa-miR-548t-3p
Chr14_9514	mml- ugagcuaaaugugucuggac hsa- ugagcuaaaugugucugga-	hsa-miR-610
Chr15_11557	mml- aaaagua <u>uu</u> cggguuuugc hsa- aaaagua <u>uu</u> cggguuuugc hsa- aaaagua <u>uu</u> cggguuuugc	*precursor mml-mir-548a hsa-miR-548l hsa-miR-548as-5p
Chr16_13228	mml- ucaacaaaaucacugaucugga mml- -caacaaaaucacugaucugga hsa- ucaacaaaaucacugaucugga	*precursor mml-mir-338 mml-miR-338-3p hsa-miR-338-3p
Chr19_17009	mml- aaagacauaggauagacuccu- hsa- aaagacauaggauagacuccu	hsa-miR-641
Chr19_17325	mml- cucuaaaaggaagcacuuuc hsa- cucuaaaaggaagcacuuuc-	*precursor mml-mir-526b hsa-miR-526b-3p
2 mismatches		
Chr8_31946	mml- aac <u>u</u> agcucugcggauccugau hsa- aac <u>u</u> agcucuguggauccuga <u>c</u>	hsa-miR-4661-5p
Chr9_34151	mml- a <u>g</u> agccaguggugagacagug- hsa- - <u>g</u> agccaguggugagacaguga	hsa-miR-4676-5p
Chr14_10835	mml- -uggcaguggaguuagugauu- mml- auggcaguggaguuagugauug hsa- auggcaguggaguuagugauug	*precursor mml-mir-34b mml-miR-34b hsa-miR-34b-3p
Chr15_12395	mml- uuccugcugaacugagccagu hsa- uuccugcugaacugagcca--	*mml precursor mir-24-1/2 hsa-miR-24-3p
Chr16_13535	mml- --ugagggccucagaccgagcuuu mml- ucugagggccucagaccgagcu-- hsa- --ugagggccagagagcgagacuuu	*precursor mml-mir-423 mml-miR-423-3p hsa-miR-423-3p
Chr16_13549	mml- --ugggacuuuguagccaguuga mml- acugggacuuuguagccaguu- hsa- acugggacuuuguagccagu--	*precursor mml-mir-193a mml-miR-193a-3p hsa-miR-193a-3p
Chr18_15585	mml- uuauauaacagccaugcacu- hsa- -uuauauaacagccaugcacu	hsa-miR-5011-5p/-3p
Chr19_16493	mml- -caguuaccgcuuccgcuaccg- hsa- ccaguuaccgcuuccgcuaccg	hsa-miR-935
Chr20_20140	mml- aaaag <u>u</u> gauugcgguuugugc mml- aaaag <u>u</u> gauugcgguuugugc	mml-miR-579 ¹
ChrX_36388	mml- aaaagcuggguugagggca- mml- aaaagcuggguugagggcgga hsa- aaaagcuggguugagggcgga	*precursor Chr8 mml-miR-320a mml-miR-320a hsa-miR-320a
>2 mismatches		
Chr1_267	mml- -cuccugccuccuugcuguaga hsa- ccuccugccuccuugcugu--	hsa-miR-1976

Chr1_788	mml- aaaaacuccaauuacuuuugu	multiple miR-548 variants
Chr2_19092	mml- -auucccuuguagauaau <u>cuggu</u> hsa- aauucccuuguagauaau <u>ccggg</u> -	hsa-miR-3938-5p
Chr2_19825	mml- ucacagugaaccggucucua <u>c</u> - mml- ucacagugaaccggucucuuuu hsa- ucacagugaaccggucucuu- -	mml-miR-128a-3p hsa-miR-128-3p
Chr4_24419	mml- uaguggaugauggagacucggu- - hsa- -aguggaugauggagacucggua <u>c</u>	hsa-miR-3691-5p
Chr6_27235	mml- --uucaguuguucaaccaguuaca mml- gguucaguguucaaccaguu- - hsa- gguucaguguucaaccaguu- -	*precursor mml-mir-582 mml-miR-585-3p hsa-miR-582-3p
Chr7_29679	mml- gaaaacuguaauuacuuuuga- - hsa- -aaaacuguaauuacuuuuga <u>ac</u>	hsa-miR-548g-3p
Chr8_32222	mml- uggcuc <u>u</u> guucagcaggagu- - mml- uggcuc <u>a</u> guucagcaggaa <u>c</u> ag hsa- uggcuc <u>a</u> guucagcaggaa <u>c</u> ag	*precursor mml-mir-24-1,-2 mml-miR-24-3p hsa-miR-24-3p
Chr14_9450	mml- aaaa <u>cc</u> caauuacuuuugc- - - mml- aaaa <u>cc</u> caauuacuuuugc- - - hsa- aaaa <u>cc</u> caauuacuuuugc <u>ac</u> ca	*precursor mml-mir-548d mml-miR-548a hsa-miR-548aa
Chr16_13311	mml- caggcugaucuucuccuuu hsa- caggcugaucuuc <u>u</u> cucu- -	hsa-miR-1253
Chr19_16436/ 17337	mml- -ucuaa <u>a</u> gggaagugcuuucuguu mml- -ucuaa <u>a</u> gggaagugcuu <u>cc</u> - -	*precursor mml-mir-518g mml-miR-518g-3p
Chr19_17321	mml- -cuc <u>a</u> aaagggaagcguuucuu- - hsa- ccuc <u>a</u> aaagggaagcguuuc <u>u</u> - hsa- ccuc <u>a</u> aaagggaagcguuuc <u>u</u> g <u>u</u> hsa- ucua <u>a</u> aaagggaagcguuuc <u>u</u> -	*precursor mml-mir-1283 hsa-miR-520f-5p hsa-miR-519d-5p hsa-miR-1283
Chr19_17329/ 17333	mml- --aaagcgcuucccuagagggg mml- agaaagcgcuucccuagag <u>g</u> - -	*precursor mml-mir-519a mml-miR-519a-5p
Chr19_17344	mml- -- <u>a</u> agca <u>cu</u> uccuuuagaggg- - mml- ag <u>a</u> agca <u>cu</u> uccuuuagag <u>g</u> - - mml- --aa <u>g</u> u <u>g</u> cu <u>cc</u> uuuagaggg <u>u</u> ac hsa- --aa <u>g</u> ca <u>cu</u> uccuuuagag <u>u</u> g- -	*precursor mml-mir-519a/b mml-miR-518g-3p mml-miR-519a-3p hsa-miR-521
Chr19_17336	mml- ----aaagugcuuccuuuggag <u>g</u> hsa- aacagaaagcgcuuccuuuggag <u>g</u>	*precursor mml-mir-519d hsa-miR-519d-5p
Chr19_17339	mml- cucuaa <u>a</u> gggaagcgcuu <u>cc</u> ua hsa- cucua <u>a</u> gggaagcgcuu <u>cc</u> u <u>g</u>	*precursor mml-mir-523b hsa-miR_523-5p
Chr19_17348	mml- --gaaagcgcuucccagaggg hsa- cagaagcgcuucccagag <u>g</u> - -	*precursor mml-mir-519b hsa-miR-519b-5p
Chrx_35401	mml- -- <u>u</u> g <u>a</u> uu <u>a</u> ca <u>ac</u> cgcu <u>a</u> ag- mml- aaug <u>a</u> uu <u>a</u> ca <u>ac</u> cgcu <u>a</u> ag- - mml- -- <u>a</u> u <u>a</u> uu <u>a</u> ca <u>ac</u> cgcu <u>a</u> ag hsa- aaug <u>a</u> uu <u>a</u> ca <u>ac</u> cgcu <u>a</u> ag-	*precursor mml-mir-374b mml-miR-374b-3p mml-miR-374b-5p hsa-miR-374b-3p

¹BLAST novel mature sequence to macaca mulatta

Table 2

Alignments and characterization of predicted novel macaque miRNA sequences. A comparison of novel macaque sequences were aligned to known human miRNAs identified through NCBI BLAST and miRBase.

Differentially Expressed Macaque Placenta miRNAs			
Mature miRNA ID	Fold Change	Mature miRNA ID	Fold Change
<i>preferentially expressed in first trimester placentas</i>			
miR-novel_chr7_29993	25.273	miR-431-5p	3.379
miR-200a-3p	19.035	miR-758-3p	3.377
miR-371-5p (mir-371-2)	14.551	miR-novel_chr7_29335	3.364
miR-371-5p (mir-371-1)	14.551	miR-432-5p	3.319
miR-149-5p	11.984	miR-654-3p	3.256
miR-376a-3p (mir-376a-2)	11.704	miR-500a-3p	3.203
miR-376a-3p (mir-376a-1)	11.704	miR-423-3p	3.139
miR-novel-chr3_22946	9.803	miR-494-3p	3.088
miR-novel-chr6_28791	9.785	miR-127-5p	3.061
miR-519a-3p	9.701	miR-1304-5p	3.018
miR-novel-chr17_14750	8.111	miR-512-3p	3.001
miR-novel-chr16_12432	8.000	miR-199a-3p	3.002
miR-934-3p	7.946	miR-novel_chr2_19386	2.949
miR-novel-chr20_20305	7.872	miR-382-5p	2.939
miR-372-3p	7.391	miR-500b-3p	2.930
miR-363-3p	6.894	miR-498-5p	2.887
miR-novel-chr20_20700	6.644	miR-323b-3p	2.869
miR-934-5p	6.525	miR-412-5p	2.859
miR-134-5p	6.468	miR-379-5p	2.752
miR-370-3p	6.253	miR-379-3p	2.682
miR-novel-chr14_10594	6.176	miR-502-3p	2.675
miR-767-5p	6.063	miR-novel_chr9_33875	2.667
miR-105-5p (mir-105-2)	5.731	miR-novel-chr4_23841	2.636
miR-105-5p (mir-105-1)	5.731	miR-146b-5p	2.608
miR-92a-3p	5.512	miR-371-3p (mir-371-2)	2.558
miR-novel-chr14_9559	5.241	miR-371-3p (mir-371-1)	2.558
miR-335-3p	4.928	miR-518a-3p (mir-518a-4)	2.555
miR-20b-5p	4.926	miR-518a-3p (mir-518a-3)	2.555
miR-224-5p	4.786	miR-518a-3p (mir-518a-1)	2.555
miR-373-3p	4.776	miR-18a-3p	2.532
miR-novel-chr2_17643	4.649	miR-130a-5p	2.476
miR-493-5p	4.616	miR-6529-5p	2.458
miR-127-3p	4.542	miR-374b-3p	2.458
miR-130b-5p	4.532	miR-378d-3p	2.385

Differentially Expressed Macaque Placenta miRNAs			
Mature miRNA ID	Fold Change	Mature miRNA ID	Fold Change
miR-92a-3p	4.436	miR-181b-5p	2.381
miR-novel-chr4_24700	4.424	miR-25-3p	2.380
miR-296-3p	4.341	miR-421-3p	2.369
miR-novel_chr18_15544	4.284	miR-181b-5p	2.365
miR-512-5p	4.182	miR-767-3p	2.358
miR-183-5p	4.124	miR-31-5p	2.354
miR-novel_chrx_36251	4.079	miR-520a-3p	2.350
miR-378a	4.043	miR-96-5p	2.337
miR-409-3p	3.951	miR-455-3p	2.321
miR-323a-3p	3.939	miR-411-3p	2.304
miR-519b-3p	3.896	miR-433-3p	2.294
miR-200a-5p	3.849	miR-518a-3p (mir-518a-2)	2.265
miR-novel_chr2_17707	3.848	miR-627-5p	2.241
miR-novel_chr2_18874	3.848	miR-1262-3p	2.235
miR-299-3p	3.832	miR-576-3p	2.222
miR-543-3p	3.777	miR-192-5p	2.198
miR-520a-5p	3.771	miR-501-3p	2.192
miR-374b-5p	3.771	miR-novel_chr9_33918	2.186
miR-654-5p	3.763	miR-452-5p	2.148
miR-519c-3p	3.760	miR-429-3p	2.133
miR-214-3p	3.670	miR-92b-3p	2.114
miR-205-5p	3.641	miR-novel_chr20_20617	2.111
miR-novel_chr19_16465	3.630	miR-342-3p	2.089
miR-182-5p	3.615	miR-197-3p	2.086
miR-novel_chr19_17320	3.609	miR-novel-chr1_609	2.080
miR-17-5p	3.570	miR-novel_chr11_6393	2.075
miR-769-5p	3.556	miR-214-5p	2.068
miR-novel-chr9_34426	3.523	miR-93-5p	2.052
miR-942-5p	3.507	miR-199a-5p (mir-199a-2)	2.047
miR-novel_chr1_123	3.495	miR-199a-5p (mir-199a-1)	2.047
miR-493-3p	3.434	miR-375-3p	2.022
miR-20a-5p	3.428	miR-576-5p	2.017
miR-novel-chr19_17336	3.423	miR-7189-3p	2
miR-106a-5p	3.415	miR-154-3p	2
miR-1180-3p	3.391		
<i>preferentially expressed in term placentas</i>			
miR-184-3p	12.091	miR-23a-3p	2.519
miR-139-5p	6.329	miR-486-5p	2.466

Differentially Expressed Macaque Placenta miRNAs			
Mature miRNA ID	Fold Change	Mature miRNA ID	Fold Change
miR-125b-5p (mir-125b-2)	5.130	miR-521-3p	2.428
miR-125b-5p (mir-125b-1)	5.109	miR-101-3p	2.412
miR-29a-3p	4.079	miR-29b-3p (mir-29b-2)	2.400
miR-100-5p	3.382	miR-29b-3p (mir-29b-1)	2.393
miR-451-5p	3.232	let7a-5p (mir-let-7a-1)	2.337
miR-146a-5p	3.210	let7a-5p (mir-let-7a-2)	2.328
miR-181a-5p (mir-181a-2)	2.999	miR-101-3p	2.326
miR-181a-5p (mir-181a-1)	2.999	let7a-5p (mir-let-7a-3)	2.310
miR-27a-3p	2.844	miR-novel_chr6_28865	2.267
miR-24-3p (mir-24-2)	2.732	miR-novel_chr4_23810	2.178
miR-24-3p (mir-24-1)	2.721	miR-518c-3p	2.103
miR-142-5p	2.719	miR-518d-3p	2.078
miR-542-3p	2.573		

Blue= C14MC miRNAs, Orange= C19MC miRNAs, Green= miR-371-3 miRNAs. Parenthesis denote the miRNA precursor variant.