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1 **Title**

2 High-throughput miRNA-sequencing of the human placenta: expression throughout gestation

3

4 **Short running title:**

5 Human placenta miRNA-seq

6

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57 (TLG, LEE, AEF, DW, YW, JT, JLC), and interpretation of the data (TLG, LEE, AEF, DW,
58 YW, CAJ, JLC, YZ, YA, HRT, KT, MDP). The original draft was written by TLG, LEE, and
59 MDP.
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61 **Disclosure of Interest**

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67 **Abstract**

68 Background: Altered placenta miRNA abundance may impact the maternal-fetal interface and
69 pregnancy outcomes. Understanding miRNA changes across gestation is essential before
70 miRNAs can be used as biomarkers or prognostic indicators during pregnancy.

71 Materials & Methods: Using next-generation sequencing, we characterize the normative human
72 placenta miRNA transcriptome in first (N=113) and third trimester (N=47).

73 Results: There are 801 miRNAs expressed in both first and third trimester, including 182 with
74 similar expression across gestation ($P \geq 0.05$) and 182 significantly different ($FDR < 0.05$). Of
75 placenta-specific miRNA clusters, C14MC is more upregulated in first trimester and C19MC is
76 more highly expressed overall.

77 Conclusion: This work provides a rich atlas of healthy pregnancies to direct functional studies
78 investigating the epigenetic differences in first and third trimester placentae.

79

80 **Lay Abstract**

81 The human body produces microRNAs which affect the expression of genes and proteins. This
82 study uses next generation sequencing to identify the microRNA profile of first and third
83 trimester human placentae using a large cohort (N=113 first, N=47 third trimester). All
84 pregnancies resulted in healthy babies. We identify microRNAs with significantly different
85 expression between first and third trimester, as well as stably expressed microRNAs. This work
86 provides a baseline for future studies which may use microRNAs to monitor maternal-fetal
87 health throughout pregnancy.

88

89 **Keywords**

90 microRNA, placenta, pregnancy, chorionic villi, gestational differences, human transcriptome

91

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92 **Introduction**

93 The placenta plays a critical role in fetal development, forming the interface between the
94 developing fetus and mother. It has multiple functions including provision of oxygen and
95 nutrients to the fetus, removal of waste products, and constitutes an important barrier protecting
96 the fetus from pathogens and environmental toxins throughout gestation. The formation and
97 development of the placenta (placentation) that begins upon implantation and continues
98 throughout the first trimester of pregnancy lays the foundation for a placenta leading to a healthy
99 gestation. After implantation, the placenta produces growth factors, cytokines and hormones
100 which target maternal physiological systems, facilitating the provision of additional blood flow
101 and nutrient delivery to the fetus.[1] During the first trimester, cytotrophoblast cells differentiate
102 into extravillous trophoblasts and syncytiotrophoblasts. Extravillous trophoblasts migrate by
103 invading and remodeling the maternal decidual extracellular matrix and spiral arteries.[2-5] This
104 occurs during a relative state of hypoxia, which promotes trophoblast invasion and
105 angiogenesis.[6-9] It is also during this time that cytotrophoblasts fuse into syncytiotrophoblasts,
106 creating a multinucleated epithelium that lines the intervillous space and also produces
107 hormones, including estrogen and progesterone for pregnancy maintenance and lactogen for fetal
108 metabolism, growth and development.[10-12] The complexities of normal placental development are
109 under the control of various epigenetic modifications that may be altered, ultimately leading to
110 abnormal placentation and adverse outcomes.[13,14]
111 MicroRNAs (miRNAs) are non-coding, single-stranded RNA molecules of about 22 nucleotides
112 in length.[15] They are important post-transcriptional regulators of gene expression. They bind to
113 RNA transcripts, causing RNA cleavage or messenger RNA translational repression, regulating
114 30-50% of all mammalian protein-coding genes.[16,17] While some miRNAs are universally
115 expressed, others are expressed preferentially or exclusively in certain tissues.[18] Two large
116 miRNA clusters are enriched in placenta, the chromosome 14 miRNA cluster (C14MC) and the
117 chromosome 19 miRNA cluster (C19MC).[19,20] C14MC is a large, imprinted, maternally-
118 expressed miRNA cluster, with several members predominantly expressed in placenta and
119 epithelial tissues.[18] C19MC is a large, imprinted, paternally-expressed miRNA cluster whose
120 members have highest expression in placenta and cancer, with relatively weak expression in
121 other tissue.[18,20-24]

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122 Widespread regulation of gene expression by miRNAs, the presence of placental-specific
123 miRNAs, and miRNA expression differences in various trophoblastic cell lines[25] suggests a role
124 for miRNAs in trophoblast behavior and placental development and function. Furthermore,
125 altered miRNA expression in the placenta may be involved in abnormal placentation and related
126 pregnancy-associated diseases, including preeclampsia[26-40] and intrauterine growth restriction
127 (IUGR).[39-43] Given that placental function changes from the first trimester during a critical state
128 of placental development and continues to function for appropriate fetal development, epigenetic
129 regulation through miRNAs likely play a key role. In addition, due to their small size and
130 stability, miRNAs are potential biomarkers of disease, particularly miRNAs from plasma
131 exosomes,[44-46] however, it is essential to know how their expression varies across gestation.
132 Previous studies comparing miRNA expression in first and third trimester placentae are limited
133 to microarray analyses and have had conflicting results.[25,47,48] We performed next-generation
134 sequencing (NGS) and expression analysis to identify and compare the normative miRNA
135 signatures in first and third trimester placentae of healthy pregnancies resulting in delivery.
136

137 **Materials and Methods**

138 *Study Population*

139 The study population consisted of 157 singleton pregnancies with available first trimester
140 placental tissue (N=110), third trimester placental tissue (N=44) or both (N=3 in both groups),
141 obtained between 2009 and 2018. Mothers with pre-existing diabetes or hypertension were
142 excluded. All subjects were enrolled under IRB approved protocols (Pro00006806,
143 Pro00008600). All pregnancies had a normal karyotype and resulted in the delivery of a viable
144 infant.

145 *Analysis of demographic data*

146 Demographic data included parental ages, races and ethnicities, maternal pre-pregnancy body
147 mass index, gestational age at chorionic villus sampling (CVS), fetal sex, maternal medical
148 history and medication use, pregnancy complications, mode of delivery, gestational age at
149 delivery, and birth weight. Means and standard deviations were reported for continuous
150 variables. Proportions were reported as percentages. Demographics were compared between
151 patients in the first and third trimester placenta, excluding 3 subjects sequenced in both groups to
152 eliminate duplicate information. T-test was used for normally distributed continuous variables,

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153 and the Wilcoxon rank-sum test was used for non-parametric data. Fisher's exact test was used
154 when appropriate. For comparison of categorical variables, the chi-square test was used.

155 *Collection of first trimester placental samples*

156 Samples from the first trimester of pregnancy were collected between 70-102 days gestation
157 during CVS procedures done for prenatal diagnosis. Samples used for research consisted of extra
158 tissue which is normally discarded after sending chorionic villi specimens for prenatal genetic
159 diagnostic testing. Fetal-derived chorionic villi (first trimester placenta tissue) were cleaned and
160 separated from any maternally-derived decidua (non-placenta tissue). Tissue samples (5-25 mg)
161 were kept on ice and submerged in RNA*later* RNA stabilization reagent (QIAGEN, Hilden,
162 Germany) within 30 minutes of collection and stored at -80°C.

163 *Collection of third trimester placental samples*

164 Samples from the third trimester of pregnancy were collected between 254-290 days gestation,
165 after delivery of a viable neonate. Samples used for research consisted of tissue which would
166 have otherwise been discarded. One centimeter cubed of placental tissue samples were obtained
167 immediately after delivery from the fetal side of the placenta near the site of cord insertion
168 beneath the amnion. The samples were cleaned and submerged in RNA*later* RNA stabilization
169 reagent (QIAGEN) and stored at -80°C.

170 *RNA extraction from first trimester placenta*

171 RNA was extracted from leftover CVS tissue utilizing a method optimized for delicate tissue.[49,50]
172 Briefly, tissue samples were thawed on ice with 600 µl of RLT Plus lysis buffer (QIAGEN)
173 containing 1% β-mercaptoethanol. Tissue was homogenized by passing at least 10 times through
174 progressively thinner gauge needles (22G, 25G, 27G) attached to an RNase-free syringe.
175 Homogenates were loaded onto AllPrep spin columns and the remainder of sample processing
176 was performed following manufacturer instructions using the AllPrep DNA/RNA/miRNA
177 Universal Kit (QIAGEN). RNA was eluted with 30-45 µl of RNase-free water at room
178 temperature and the elution was passed through the column twice to improve yields, as
179 previously described.[49,50] The average RNA integrity number (RIN) for sequenced first trimester
180 placenta samples was 8.87.

181 *RNA extraction from third trimester placenta*

182 Third trimester placenta tissue was thawed on ice, then a quarter of collected tissue was diced
183 with RNase-free blades coated in RNA*later* buffer, tissue was sonicated on ice in lysis buffer

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184 (600 µL RLT Plus lysis buffer with 1% β-mercaptoethanol) using 5 second pulses on a low
185 setting (#2) until tissue fragments were small enough to complete homogenization with RNase-
186 free needles. Further extraction was performed as described for first trimester tissue. The average
187 RIN for sequenced third trimester placentae was 8.84.

188 *Library preparation and miRNA sequencing*

189 A miRNA sequencing library was prepared from total RNA using the QIASeq miRNA Library
190 Kit (QIAGEN, Hilden, Germany). A pre-adenylated DNA adapter was ligated to the 3' ends of
191 miRNAs, followed by ligation of an RNA adapter to the 5' end. A reverse-transcription primer
192 containing an integrated Unique Molecular Index (UMI) was used to convert the 3'/5' ligated
193 miRNAs into cDNA. After cDNA cleanup, indexed sequencing libraries were generated via
194 sample indexing during library amplification, followed by library cleanup. Libraries were
195 sequenced on a NextSeq 500 (Illumina, San Diego, CA) with a 1x75 bp read length and an
196 average sequencing depth of 10.64 million reads per sample.

197 *Differential expression analysis of miRNAs*

198 The demultiplexed raw reads were uploaded to GeneGlobe Data Analysis Center (QIAGEN) at
199 https://www.qiagen.com/us/resources/geneglobe/ for quality control, alignment and expression
200 quantification. Briefly, 3' adapter and low quality bases were trimmed off from reads first using
201 *cutadapt* v1.13 with default settings,[51] then reads with less than 16bp insert sequences or with
202 less than 10bp UMI sequences were discarded. The remaining reads were collapsed to UMI
203 counts and aligned sequentially to miRBase release 21 mature and hairpin RNA databases using
204 *Bowtie* v1.2.[52,53] The UMI counts of each miRNA category were quantified, and then normalized
205 by a size factor-based method in the R package *DESeq2* v1.22.2 (Bioconductor).[54] Data were
206 averaged across all samples in each group (first and third trimester) for each respective miRNA
207 and were reported as baseMean. The R package *FactoMineR* v1.41 was used to conduct principal
208 components analysis (PCA), which was used to investigate clustering and potential outliers.
209 Differential expression analysis was performed with *DESeq2* to compare first versus third
210 trimester expression, adjusting for fetal sex. Each miRNA was fitted into a negative binomial
211 generalized linear model, and the Wald test was applied to assess the differential expressions
212 between two sample groups (P value). The Benjamini and Hochberg procedure was applied to
213 adjust for multiple hypothesis testing, and those with false discovery rate (FDR) less than 0.05
214 were selected as significantly differentially expressed miRNAs. The genome locations of

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215 miRNAs were identified by cross-referencing mature miRNA IDs with precursor miRNA
216 accession IDs in miRBase release 21, then using R package *biomaRt* v2.45.8 and Ensembl
217 release 91 (which contains miRBase release 21) to retrieve chromosome locations.[55,56] For
218 miRNAs derived from more than one precursor, all chromosomal locations were counted in bar
219 plots (e.g. hsa-miR-1184 is encoded by three precursors on chromosome X, thus was counted
220 three times) and each miRNA was plotted once per chromosome in scatter plots of genome
221 distribution.

222 *Analysis of miRNA expression*

223 Counts normalized for sequencing depth (baseMeans) were used as a measure of expression
224 since miRNA lengths do not vary substantially. All expressed miRNAs were defined as any with
225 baseMean>10 in both first or third trimester placenta groups. The miRNAs common in both first
226 and third trimester placentae, also known as similarly expressed, were defined by P≥0.05,
227 absolute fold-change≤2, and baseMean>10 in both trimesters. The P≥0.05 threshold excludes all
228 significantly different miRNAs (FDR<0.05) as well as other potentially different miRNAs
229 (P<0.05). Differentially expressed miRNAs were defined as FDR<0.05, absolute fold-change>2,
230 and baseMean>10 in both trimesters. Higher expression thresholds were selected for target
231 enrichment analysis when needed (next section). For miRNAs in C14MC and C19MC, these
232 filters were applied: baseMean>1 and P≥0.05 (similarly expressed) or baseMean>1 and
233 FDR<0.05 (differentially expressed).

234 *Enrichment analysis of predicted miRNA target genes*

235 Ingenuity Pathways Analysis (IPA) software's microRNA Target Filter application (QIAGEN,
236 Redwood City, CA, USA, http://www.qiagenbioinformatics.com/IPA) was used to generate a list
237 of target RNAs based on sequence and experimental confirmation. Targets were included if
238 biochemically confirmed using human tissue or non-species methods (sourced from QIAGEN's
239 curated Ingenuity Knowledge Base, or the publicly available miRecords or TarBase). IPA's Core
240 Analysis function was used to test the hypothesis that the target genes are enriched in canonical
241 biological pathways, as previously described.[49,57,58] Supplemental data also show Core Analysis
242 results with additional targets predicted with high confidence according to IPA, based on the
243 TargetScan algorithm previously described.[59] IPA designates high confidence as a cumulative
244 weighted context score of -0.4 or lower, predicting decreased expression by at least 25% due to a
245 specific miRNA.

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246 The input miRNAs were reduced with higher expression thresholds so that target gene numbers
247 did not exceed IPA software limitations for Core Analysis. The following definitions were
248 applied for highly expressed miRNAs (baseMean>10,000 in both trimesters), similarly expressed
249 miRNAs ($P \geq 0.05$, absolute fold-change≤2, and baseMean>1,000 in both trimesters), and
250 differentially expressed miRNAs (FDR<0.05, absolute fold-change>2, and baseMean>1,000 in
251 both trimesters). Due to their smaller number, no additional miRNA filters were required for
252 Core Analysis of C14MC and C19MC targets.

253 *Heatmaps*

254 Heatmap and dendrograms of samples versus miRNAs were created with a matrix of
255 $\log_2$(baseMean) values scaled and centered by rows. The heatmaps and dendrograms were
256 created with hierarchical clustering from R package *gplots* v3.1.1. Heatmaps of gene enrichment
257 were created with R package *pheatmap* v1.0.12 with a matrix of $-\log_{10}(P)$ output from IPA Core
258 Analysis.

259 *Validation with qRT-PCR*

260 Expression of 6 selected miRNAs was re-analyzed with an independent cohort by qRT-PCR
261 using the miRCURY LNA miRNA PCR system (QIAGEN). The 6 selected (hsa-miR-144-3p,
262 hsa-miR-24-3p, hsa-miR-126-3p, hsa-miR-145-5p, hsa-miR-143-3p, hsa-miR-126-5p) had high
263 expression (baseMean>1000) and were significantly different in miRNA-sequencing ($FDR<10^{-13}$)
264 between first and third trimester. A highly expressed miRNA with stable expression in first
265 and third trimester placentae was used as a reference gene (hsa-miR-130a-3p; baseMean>10,000
266 in both trimesters, P=0.9693, FDR=0.9889). RNA from first trimester (N=10) and third trimester
267 (N=6) placenta samples were extracted, then cDNA synthesized using universal primers in the
268 miRCURY LNA RT Kit (QIAGEN). Expression was quantified by qRT-PCR using the
269 miRCURY LNA SYBR Green PCR Kit (QIAGEN) and a BioRad MyIQ machine, analyzed
270 using the $\Delta\Delta$Ct method,[60] with hsa-miR-130a-3p as an internal reference. Statistics were
271 performed using the Wilcoxon rank-sum test on ΔCt values.

272

273 **Results**

274 *Cohort demographics*

275 There were N=113 first trimester placenta samples and N=47 third trimester placenta samples
276 studied, including 3 subjects with both first and third trimester placenta sequenced. Principal

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277 components analysis (PCA) shows that first and third trimester placenta segregated into distinct
278 clusters along PC1 (29.27% variability explained) and PC2 (20.22% variability explained)
279 (Supplemental File 1). There were significantly more non-Hispanic, Caucasian parents and
280 fetuses in the first trimester group (Table 1). However, race and ethnicity groups did not cluster
281 in PCA analyses of the miRNA transcriptome (Supplemental File 2). Maternal pre-pregnancy
282 BMI and thyroid disorders requiring thyroid replacement were significantly different among the
283 groups (Table 1). There were more cases of pregnancy-induced hypertension requiring
284 antihypertensives and/or magnesium in the third trimester placenta group, compared to none in
285 the first trimester placenta group.

286 *All expressed miRNAs in placenta*

287 We identified 2503 mature miRNAs with high-throughput sequencing of first and third trimester
288 placentae, with 801 miRNAs reaching 10 normalized counts in both first and third trimester
289 placentae (baseMean>10). First trimester placenta expressed 872 mature miRNAs
290 (baseMean>10), derived from 967 miRNA precursors from all chromosomes with annotated
291 miRNAs (22 autosomes and the X chromosome) (Figure 1A, Supplemental File 3). The majority
292 of these precursor miRNAs originate from chromosomes 19 (12.7%), 14 (10.8%), X (9.6%), and
293 1 (6.8%). Third trimester placenta expressed 882 mature miRNAs (baseMean>10), derived from
294 985 miRNA precursors from all 22 autosomes and the X chromosome (Figure 1A, Supplemental
295 File 3). The most represented chromosomes were also 19 (12.4%), 14 (10.5%), X (9.7%), and 1
296 (7.1%).

297 *Highly expressed miRNAs in placenta*

298 Some miRNAs had expression values several orders of magnitude higher than most miRNAs,
299 with the median at baseMean=123.6 but the mean raised to baseMean=5,635 by these highly
300 expressed miRNAs (Supplemental File 3). A threshold of baseMean>10,000 was selected for the
301 "most highly expressed" miRNAs. There were 75 mature miRNAs (derived from 96 precursors)
302 in first trimester and 77 mature miRNAs (derived from 97 precursors) in third trimester placenta
303 which reached this threshold. The most highly expressed miRNA in first trimester was C19MC
304 member miR-517b-3p (baseMean=218,953). The most highly expressed miRNA in third
305 trimester and overall most highly expressed was miR-126-3p (baseMean=337,399).
306 Chromosome 19 encoded 30 mature miRNAs (derived from 36 precursors) which reached
307 baseMean>10,000 in both first and third trimester (Figure 1B), making chromosome 19 the

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308 source of over 37% of the most highly expressed precursor miRNAs in human placenta.
309 Specifically, 28 of the 36 precursor miRNAs were C19MC members, and 8 localized elsewhere
310 on chromosome 19. The next chromosomes contributing the most highly expressed miRNAs
311 were chromosome 9, chromosome 1, and chromosome X.
312 We performed pathway enrichment analysis on experimentally confirmed targets of the most
313 highly expressed miRNAs (Figure 1C, Supplemental File 5Ai). The most significantly enriched
314 canonical pathways in first and third trimester were "Molecular Mechanisms of Cancer",
315 "Hepatic Fibrosis Signaling", "Senescence", "Regulation of the Epithelial Mesenchymal
316 Transition by Growth Factors", and "Pancreatic Adenocarcinoma Signaling."
317 Pathway enrichment analysis with both experimentally confirmed miRNA targets as well as
318 targets predicted with high confidence demonstrated similar patterns, though third trimester
319 showed relatively higher enrichment in "Hepatic Fibrosis/Hepatic Stellate Cell Activation" and
320 "Regulation of the Epithelial Mesenchymal Transition by Growth Factors" compared to first
321 trimester (Supplemental File 4, Supplemental File 5Ai). Additional pathways, including
322 inflammatory pathways such as "Neuroinflammation", "Prolactin", "Systemic Lupus
323 Erythematosus in B Cell", and "IL-6" signaling were also enriched (Supplemental File 5Aii).
324 *Similarly expressed miRNAs in first and third trimesters*
325 There were 182 mature miRNAs with similar expression in the first and third trimester placentae
326 ($P \geq 0.05$, fold-change≤ 2 and baseMean>10, Supplemental File 3), suggesting consistent
327 expression throughout gestation (Figure 1D). These mature miRNAs are derived from 206
328 precursor miRNAs, with greatest representation from chromosomes 19 (17.0%), 14 (10.2%), X
329 (9.7%), and 1 (7.3%) (Figure 1E). The most highly expressed similar miRNA was C19MC
330 member hsa-miR-515-5p with first trimester baseMean=129,659 and third trimester
331 baseMean=129,323, P=0.902 between trimesters. This was followed closely by other C19MC
332 members: hsa-miR-158b, hsa-miR-518f-3p, hsa-miR-1323, and hsa-miR-1283.
333 *Differentially expressed miRNAs between first and third trimesters*
334 There were 588 mature miRNAs significantly differentially expressed between first and third
335 trimester placentae (FDR<0.05, baseMean>10) (Figure 2A), further filtered to 180 miRNAs with
336 fold-change>2, including 91 upregulated in the first and 89 upregulated in the third trimester
337 (Figure 2B, Supplemental File 3). The 180 differentially expressed miRNAs were derived from
338 202 precursors with highest representation from chromosomes 9 (9.9%), X (9.4%), 1 and 14

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339 (8.4% each), and 19 (7.4%) (Figure 2B). The most differentially expressed miRNA was hsa-
340 miR-4483, with 38.2-fold higher expression in the first trimester placenta (FDR=0) and a
341 baseMean decrease from 984.1 to 25.5 from first to third trimester (Figure 2C, Supplemental File
342 3). The next most significantly differentially expressed miRNA was hsa-miR-139-5p with 18.1-
343 fold higher expression in the third trimester (FDR=3.69×10^{-298}), baseMean increasing from 28.0
344 to 497.5 (Figure 2C). The differentially expressed miRNA with the highest overall expression
345 was hsa-miR-126-3p, with a 3.13-fold higher expression (FDR=4.52×10^{-97}) in third trimester
346 placenta (baseMean=337,399) compared to first trimester placenta (baseMean=107,787) (Figure
347 2D). Of the differentially expressed miRNAs, those with the greatest fold changes had lower to
348 moderate expression around baseMean 100-1,000 and were predominantly elevated in the first
349 trimester, whereas those with the highest expression had lower fold changes and were
350 predominantly elevated in the third trimester (Figure 2D). Zero differentially expressed miRNAs
351 with baseMean>1,000 reached fold-change of 4 (Figure 2D, Supplemental File 3).

352 *Validation of differentially expressed miRNAs*

353 Six differentially expressed miRNAs identified using NGS were selected for validation (Figure
354 2E). We performed qRT-PCR using an independent cohort of first (N=10) and third trimester
355 (N=6) placenta samples. The miRNA hsa-miR-130a-3p was selected as an internal reference due
356 to high and stable expression in first and third trimester placentae (P=0.9693, fold-
357 change=0.9984 first/third, baseMean=11,097). All six validated miRNAs (hsa-miR-24-3p, hsa-
358 miR-144-3p, hsa-miR-145-5p, hsa-miR-126-3p, hsa-miR-126-5p, and hsa-miR-143-3p) were
359 upregulated in third trimester placenta with 2.5 to 3.7-fold changes by sequencing, and all six
360 were confirmed significant by qRT-PCR with P<0.003 (Figure 2E).

361 *Comparison of similarly and differentially expressed miRNAs*

362 A heatmap of the 182 similarly expressed miRNAs shows no clustering of the first and third
363 trimester samples (Figure 3A). The heatmap of 180 differentially expressed miRNAs shows
364 placenta sample clustering by trimester, and miRNAs clustering into two groups by direction of
365 upregulation (Figure 3B). There was little subject variability in miRNAs in first and third
366 trimester, but some miRNAs were not consistently expressed (baseMean=0, red).

367 Pathway enrichment analysis was performed for experimentally confirmed miRNA targets to
368 identify potential regulatory roles of the miRNAs expressed in placenta (Figure 3C,
369 Supplemental File 4B). The most significantly enriched pathways, targeted by both similarly and

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370 differentially expressed miRNAs in first and third trimester placenta were "Molecular
371 Mechanisms of Cancer" and "Hepatic Fibrosis Signaling". None of the top 20 pathways were
372 more significantly targeted by similarly expressed miRNAs, suggesting high variability
373 throughout gestation (Supplemental File 5Bi). Differentially expressed miRNAs targeted more
374 significantly by highly expressed miRNAs in the first trimester include "Molecular Mechanisms
375 of Cancer", "Hepatic Fibrosis Signaling", "Senescence", and "Regulation of the Epithelial
376 Mesenchymal Transition by Growth Factors" pathways, suggesting these pathways are distinctly
377 regulated by miRNAs in the first versus third trimester (Figure 3C, Supplemental File 5Bi).
378 When the pathway enrichment analysis was repeated with both experimentally confirmed
379 miRNA targets as well as targets predicted with high confidence, additional patterns emerge.
380 Differentially expressed miRNAs target the "Hepatic Fibrosis / Hepatic Stellate Cell Activation"
381 pathway more heavily than similar miRNAs when predicted targets are included (Supplemental
382 File 5Bii). Addition of predicted targets highlights specific cytokine and growth factor pathways,
383 including "IL-6" and "IGF-1" signaling which are heavily targeted by similarly expressed
384 miRNAs, and less so by differentially expressed miRNAs (Supplemental File 4, Supplemental
385 File 5Bii).
386 *Expression from C14MC and C19MC*
387 The placenta specific miRNA clusters expressed 42 mature miRNAs similarly expressed
388 between first and third trimester placenta ($P \geq 0.05$, baseMean>1), 24 from C14MC and 18 from
389 C19MC (Figure 4AB, Supplemental File 6). There were 105 mature miRNAs differentially
390 expressed between first and third trimester ($FDR<0.05$ and baseMean>1), 64 from C14MC and
391 41 from C19MC (Figure 4AB, Supplemental File 6). The cluster miRNAs with highest fold-
392 change came from C14MC: hsa-miR-1197 (6.28-fold, $FDR=4.83 \times 10^{-118}$), hsa-miR-758-5p (5.59-
393 fold, $FDR=5.07 \times 10^{-101}$), hsa-miR-496 (4.05-fold higher in first, $FDR=3.92 \times 10^{-109}$), and hsa-miR-
394 665 (3.98-fold, $FDR=1.75 \times 10^{-95}$), all higher in first trimester compared to third trimester
395 placenta.
396 The most significantly upregulated third trimester miRNA was hsa-miR-520c-3p (2.78-fold
397 higher, $FDR=2.91 \times 10^{-111}$), followed by hsa-miR-181d-5p (3.18-fold higher, $FDR=6.38 \times 10^{-70}$),
398 both from C19MC. Overall, the C14 miRNA cluster contributed more differentially expressed
399 miRNAs, reaching higher fold-changes in first trimester than C19MC (Figure 4A). Although
400 C19MC contributed fewer total miRNAs, and at lower fold-change differences between

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401 trimesters, the C19MC baseMean distribution was an order of magnitude higher than C14MC
402 distribution in both overall baseMean median (C19MC=5,696; C14MC=198.7) and mean
403 (C19MC=21,278; C14MC=1,122) (Figure 4B, Supplemental File 6). This held true for both first
404 and third trimester baseMeans expression values. Pathway enrichment analysis of experimentally
405 confirmed target genes shows that distinct pathways are regulated by similarly and differentially
406 expressed miRNAs (Figure 4Ci). Metabolite salvage pathways and GTPase signaling were more
407 significantly targeted by differentially expressed C14MC miRNAs, including "Pyridoxal 5'-
408 phosphate Salvage Pathway", "Salvage Pathways of Pyrimidine Ribonucleotides", and
409 "Signaling by Rho Family GTPases" suggesting these pathways are uniquely regulated by this
410 cluster miRNAs between the first and third trimester. "Prostate Cancer Signaling" was more
411 significantly targeted by the similarly expressed C14MC miRNAs, suggesting this pathway is
412 important throughout gestation (Supplemental File 5C). "Molecular Mechanisms of Cancer",
413 "Pancreatic Adenocarcinoma Signaling", "Prostate Cancer Signaling", "Estrogen-mediated S-
414 phase Entry", and "Chronic Myeloid Leukemia Signaling" were more significantly targeted by
415 differentially expressed C19MC miRNAs suggesting these pathways are uniquely regulated by
416 this cluster miRNAs between the first and third trimester. Conversely, there were no pathways
417 among the top 30 most enriched that were targeted by similarly expressed C19MC miRNAs,
418 suggesting more variable regulation throughout gestation (Figure 4Cii, Supplemental File D).
419 Pathway enrichment analysis with both experimentally confirmed miRNA targets as well as
420 targets predicted with high confidence (Supplemental File 5D), demonstrated changes in
421 regulated pathways, with greater representation of inflammatory pathways, including the
422 "Systemic Lupus Erythematosus in B Cell Signaling" and "Coronavirus Pathogenesis" pathways,
423 as well as several pathways more significantly targeted by similarly expressed miRNAs,
424 including "Role of PI3K/AKT Signaling in the Pathogenesis of Influenza".
425
426 **Discussion**
427 The placenta is a unique organ that changes function greatly throughout gestation, meeting
428 different challenges and needs at different stages of pregnancy. Placentation in the first trimester
429 sets the groundwork for its functions throughout gestation for fetal development. Placental
430 function is in part epigenetically regulated through miRNAs, including the placenta-specific
431 miRNA clusters, C14MC and C19MC that play critical roles in regulation of this vital organ.

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432 This is the first study to our knowledge to use high-throughput sequencing to compare miRNA
433 expression between first and third trimester human placentae of healthy pregnancies resulting in
434 delivery.
435 The miRNA expression profiles in first and third trimester have similar chromosome
436 distributions, with expected peaks at chromosomes 14 and 19, as well as peaks at chromosome 1,
437 the largest human chromosome and chromosome X, which has a higher density of miRNAs
438 compared to autosomes.[61,62] The most highly expressed miRNA, hsa-miR-126-3p, was
439 upregulated in third trimester, and was validated with qRT-PCR using an independent cohort. In
440 a recent study comparing first and second trimester placenta, miR-126-3p was identified to be
441 among the 10 most highly expressed miRNAs and identified in maternal plasma.[63] Although
442 variation among the first and second trimester was not different,[63] our study identified hsa-miR-
443 126-3p to be differentially expressed and highest in the third trimester, likely having a unique
444 role in the third trimester compared to earlier in gestation. It is also highly abundant in fetal
445 circulation and human umbilical vein endothelial cells,[64] suggesting a role during parturition and
446 fetal development and may become a potential biomarker for developmental origins of health
447 and disease.
448 Among the most highly expressed miRNAs, over 37% were encoded in chromosome 19 (and
449 28/36 or 77.8% were specifically in C19MC), whereas none localized to chromosome 14. This
450 supports an earlier miRNA-seq study which profiled 25 human placentae at delivery and
451 identified higher expression from C19MC than C14MC miRNAs[65], and additionally we show the
452 same pattern in first trimester. The C19MC miRNAs with high expression in the placenta may
453 potentially be used for targets, as C19MC miRNAs have been identified in maternal circulation,
454 as early as the first trimester, with elevations throughout gestation.[66-68] The most highly
455 expressed miRNA that was similarly expressed was hsa-miR-515-5p, which is a member of the
456 C19MC. Placental expression of hsa-miR-515-5p has been identified to play a key role in human
457 trophoblast differentiation with aberrant up-regulation contributing to pathogenesis of
458 preeclampsia.[35,69] It has also been associated with preterm birth[70] and fetal growth restriction.[43]
459 Although, it has been detected in maternal circulation, both in plasma and whole blood fractions,
460 it has also been detected in whole blood fractions of healthy nonpregnant women,[71] and may not
461 be used solely as a biomarker of disease, but may be incorporated with other miRNAs with

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462 stable expression across gestation that change with disease using a bivariate biomarker disease
463 approach described by Laurent.[45]
464 This atlas identified 180 differentially expressed miRNAs which may be important for functional
465 changes in the placenta throughout pregnancy. Among those, the most differentially expressed
466 miRNAs were highest in the first trimester. The most significantly targeted pathways of
467 experimentally confirmed targets by differentially expressed miRNAs and the most highly
468 expressed in the first trimester was "Molecular Mechanisms of Cancer." Although identified in
469 pathway enrichment analyses for tumor progression, many of the major signaling pathways
470 involved in inter- and intra-cellular communication of invasive phenotypes mimic those
471 associated with migration and invasion of trophoblasts into the maternal decidua and spiral
472 arteries. These essential placentation steps take place in an environment rich in hormones,
473 cytokines, and growth factors and include responsible signaling pathways such as mitogen-
474 activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt),
475 Janus kinase (JAK)/signal transducer and activator of transcription proteins (STATs), wingless
476 (Wnt), and focal adhesion kinase (FAK) pathways.[2] Of these miRNAs, the most differentially
477 expressed with highest expression in first trimester, hsa-miR-4483, was also found to be strongly
478 downregulated in the second trimester and hence likely plays a significant role in the first
479 trimester placenta. It may function to regulate estradiol production early in gestation, as
480 described in another hormone producing cell type and contribute to migration and invasion.[72]
481 Differentiation of first trimester human placental cytotrophoblasts from an anchorage dependent
482 epithelial phenotype into the mesenchymal-like invasive extravillous trophoblast is a crucial step
483 for placentation. Illsley et al, previously demonstrated that an epithelial to mesenchymal
484 transition takes place when first trimester cytotrophoblasts differentiate into extravillous
485 trophoblasts.[73,74] MiRNAs have been implicated in the epithelial to mesenchymal transition.[75-80]
486 The most highly differentially expressed miRNA in first trimester placenta, hsa-miR□205 has
487 been implicated in the epithelial to mesenchymal transition and the maintenance of the epithelial
488 phenotype.[75-79] In human trophoblast cell lines it has been identified to silence MED1 under
489 hypoxic conditions.[75] suggesting it has a role in the first trimester regulating trophoblast
490 differentiation during physiologic hypoxic conditions.[75,81,82] "Regulation of the Epithelial
491 Mesenchymal Transition by Growth Factors Pathway" was also one of the most significantly
492 targeted pathway by differentially expressed miRNAs and most highly expressed in the first

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493 trimester, highlighting the differences between placentation when the placenta is invading
494 maternal tissue and establishing itself in states of low oxygen tension versus time of delivery
495 when the placenta has completed its purpose.
496 Two additional significantly targeted pathways by differentially expressed miRNAs and highly
497 expressed in the first trimester included the "Hepatic Fibrosis Signaling" and the "Senescence"
498 pathways. Hepatic Fibrosis Signaling is classically associated with extracellular matrix
499 deposition,[83] consistent with first trimester placental function when extravillous trophoblasts
500 degrade and induce ECM remodeling to enable migration.[2,5,84,85]
501 Cellular senescence is programmed cell-cycle arrest that restricts the propagation of cells, which
502 is induced by various forms of cellular stress, including oxidative stress. Cell fusion, has also
503 been identified to trigger cellular senescence and has been described in the placenta, with the
504 placental expressed fusogen, Syncitin-1 (*ERVWE1)*, which mediates cell-fusion-induced
505 senescence of the syncitiotrophoblast.[86-88] These senescent cells secrete inflammatory cytokines,
506 chemokines and matrix metalloproteinases, known as the senescence associated secretory
507 phenotype (SASP). SASP proteins promote EMT and the degradation of basement membranes,
508 increasing migration and invasion for appropriate placentation.[89]
509 Our findings also support the importance of the placenta-specific miRNA clusters throughout
510 gestation, with 42 miRNAs similarly expressed and 105 differentially expressed across first and
511 third trimester. This indicates that while they are placenta-specific miRNAs, the majority have
512 varying roles throughout pregnancy. Differentially expressed canonical pathways targeted by the
513 C14 and C19 clusters were more significant than those of all similarly expressed miRNAs
514 suggesting these clusters have significantly different roles throughout gestation.[66] Similar to
515 other studies using whole villous tissue and primary cytotrophoblasts,[20,48,63] we identified a
516 decrease in C14MC expression from first to third trimester. However, a recent study did not
517 identify a decrease throughout gestation, but their study only focused on the first and second
518 trimester of presumably normal pregnancies.[63]
519 The major strengths of this study are the use of first and third trimester tissue from healthy
520 pregnancies resulting in delivery, the cohort size, the availability of detailed demographic
521 information, and the use of high-throughput sequencing. NGS, as opposed to other techniques
522 such as array, allows for greater confidence in the conclusions regarding differential expression,
523 since all known miRNA species previously annotated in the human genome are considered, and

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524 bias is not introduced by eliminating certain RNAs. Previous studies analyzing miRNA
525 expression in first and/or third trimester placentae have used microarray technology and most
526 examined very few samples (N=2-6 in each group).[25,47,48,90] There are currently few NGS
527 miRNA profiles of the placenta, and our study is the first to profile both first and third trimester
528 placentae with NGS and a large sample size. We successfully validated all six selected miRNAs
529 using qRT-PCR and an independent cohort.
530 Our study has some limitations. There were some differences in the demographics between the
531 groups from the first and third trimester placenta samples. This includes race, ethnicity, maternal
532 BMI, thyroid disorders, and pregnancy complications, specifically hypertension. However, the
533 overall differences were small. In addition, PCA analysis did not demonstrate outliers.
534 Furthermore, we performed pathway enrichment analysis using only experimentally confirmed
535 targets. When performed using both experimentally confirmed and predicted with high
536 confidence targets, although overall pathways and patterns remained consistent, when we only
537 included experimentally confirmed targets, immune mediated pathways were not represented.
538 Overall, we intended to identify and compare the normative miRNA signatures in the first and
539 third trimester placentae. Our study shows many stably expressed miRNAs throughout gestation
540 as well as significant differences between the miRNA signatures. This work provides a rich atlas
541 to direct functional studies investigating the epigenetic differences in first and third trimester
542 placentae and development of disease related biomarkers or prognostic indicators that are
543 gestational age specific.
544
545 **Future Perspective**
546 As we improve our understanding of miRNA profiles in placenta and across gestation, miRNAs
547 may be useful biomarkers for non-invasive prenatal diagnostic testing. Our knowledge of
548 miRNA profiles is still in its infancy relative to our knowledge of the protein coding
549 transcriptome. Until recently, most miRNA profiling papers of placenta used arrays with limited
550 samples. However, protocols to capture small RNAs, synthesize cDNA, and perform high-
551 throughput NGS are improving rapidly. In 5-10 years' time, we expect that the knowledge of
552 human miRNA profiles in different tissue and extracellular locations will greatly improve as
553 well. This will provide opportunities for biomarker discovery and diagnostic test development,
554 since miRNAs are smaller, more stable RNAs than protein coding transcripts. Currently, the

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555 knowledge pool of miRNA targets has limited confirmed miRNA-RNA interactions, but this will
556 improve as the miRNA field continues to evolve. Our work to profile miRNAs in first and third
557 trimester provides a foundation for biomarker discovery during pregnancy and future
558 advancements in maternal-fetal health.
559

560 **Executive Summary**

561 • This work creates an atlas of the miRNA expression profiles of first and third trimester
562 human placenta from patients who delivered healthy babies.
563 • Chromosome 19 contributes approximately 37% of the most highly expressed miRNAs in
564 both first and third trimester placenta. Most of these miRNAs are localized to the pregnancy-
565 associated miRNA cluster, C19MC.
566 • There are 182 miRNAs with similar expression across gestation. Other patient variables may
567 affect the abundance of these miRNAs.
568 • There are 180 miRNAs with significant differences in expression between first and third
569 trimester placenta. These miRNAs may contribute to changes in placental function or be
570 markers of different placental stresses throughout gestation.
571 • Six miRNAs were successfully validated with qRT-PCR in an independent cohort.
572 • The placenta-specific miRNA clusters (C14MC and C19MC) contain both similarly and
573 differentially expressed miRNAs.
574 • C14MC expressed miRNAs with greater fold-change differences across gestation than
575 C19MC miRNAs, though C14MC miRNAs are not among the most highly expressed
576 miRNAs in placenta.
577 • For both similarly and differentially expressed miRNAs, C19MC miRNA placenta
578 expression was overall higher than C14MC expression.

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579 **Figure/Table Legends**

580 Table 1 - Demographics

	First Trimester	**Third Trimester**	**P value**
N	110	44	
Maternal age, years	37.7 (3.0)	37.3 (3.0)	0.65
Paternal age, years	39.5 (4.8)	38.6 (4.8)	0.30
Maternal race/ethnicity			
Caucasian	106 (96.4%)	35 (79.6%)	0.002
Non-Hispanic	107 (97.3%)	35 (79.6%)	<0.001
Paternal race/ethnicity			
Caucasian	104 (94.6%)	37 (84.1%)	0.04
Non-Hispanic	107 (97.3%)	36 (81.8%)	0.001
Fetal race/ethnicity			
Caucasian	103 (93.6%)	32 (72.7%)	<0.001
Non-Hispanic	106 (96.4%)	33 (75.0%)	<0.001
Fetal female sex	57 (51.8%)	18 (40.9%)	0.22
Maternal pre-pregnancy BMI, kg/m^2	21.9 (3.4)	24.0 (4.7)	0.005
Maternal pre-existing medical conditions			
Hypertension	0 (0%)	0 (0%)	-
Diabetes	0 (0%)	0 (0%)	-
Thyroid disorder	3 (2.7%)	6 (13.6%)	0.02
Pregnancy complications			
Hypertension (not pre-existing)	0 (0%)	8 (18.2%)	<0.001
Gestational diabetes	3 (2.7%)	1 (2.3%)	1.0
Placenta previa	0 (0%)	1 (2.3%)	0.49
Placental abruption	0 (0%)	0 (0%)	-
Hypertension management in pregnancy			
Anti-Hypertensives	0 (0%)	3 (6.8%)	0.022
Any Magnesium use (Ante- or Postpartum)	0 (0%)	6 (13.6%)	<0.001
Mode of delivery- Cesarean section	33 (30%)	15 (34.1%)	0.74
Gestational age at delivery, days	276.3 (7.0)	276.5 (8.0)	0.43
Birthweight, g	3435.4 (463.6)	3473.1 (467.0)	0.7

581 Note: Values shown as mean (standard deviation) or n (%). P values were adjusted for fetal sex.

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582

583 **Figure 1. Expressed miRNAs in first and third trimester placenta.** (A, B) The chromosome
584 distribution of miRNAs expressed in placenta at first trimester (N=113 samples) and third
585 trimester (N=47 samples). Bar plots count all genomic locations (all precursor miRNAs)
586 corresponding to mature miRNAs identified through sequencing, at thresholds: (A) all expressed
587 with baseMean>10 or (B) the most highly expressed with baseMean>10,000. (C) Pathway
588 enrichment analysis with experimentally confirmed target genes of the most highly expressed
589 miRNAs. (D) The expression distribution of miRNAs similarly expressed in first and third
590 trimester at P≥0.05 and FC≤2. The red line (baseMean=10) is the threshold selected for stable
591 expression. (E) Counts of similarly expressed miRNAs with P≥0.05, FC≤2, baseMean>10 in
592 both trimesters.

593 **Figure 2. Differentially expressed miRNAs between first and third trimester placenta.** (A)
594 Scatter plot of absolute fold-change distribution across chromosomes for all differentially
595 expressed (DE) miRNAs at FDR<0.05 and baseMean>10. The dotted line represents FC=2. (B)
596 Chromosome frequency of 180 DE miRNA precursors at FDR<0.05, FC>2, baseMean>10. (C)
597 Volcano plot of all miRNAs with baseMean>10. Key as in A, with addition of open black
598 squares for non-significant miRNAs (FDR≥0.05). (D) Expression versus absolute fold-change
599 for 180 DE miRNAs. (E) Six DE miRNAs (green) were selected for validation via qRT-PCR in
600 an independent cohort. The bar plot shows qRT-PCR results normalized to an internal reference,
601 hsa-miR-130a-3p (blue). The superimposed line shows fold-changes in miRNA-seq. All six
602 miRNAs were validated significantly different between first and third trimester with P<0.003.

603 **Figure 3. Heatmaps showing sample miRNA variability.** Heatmaps: rows = scaled and
604 centered miRNA $\log_2$(baseMean), columns = hierarchically clustered samples. BaseMean=0
605 samples are highlighted red. (A) 182 similarly expressed miRNAs with P≥0.05, FC≤2, and
606 baseMean>10. The miRNAs are listed alphabetically. (B) 180 differentially expressed miRNAs
607 with FDR<0.05, FC>2, and baseMean>10. The miRNAs are hierarchically clustered. (C)
608 Pathway enrichment analysis for experimentally confirmed targets of similarly (S) and
609 differentially (D) expressed miRNAs.

610 **Figure 4. Placenta-specific C14MC and C19MC.** (A,B) Expression versus absolute fold-
611 change plots for cluster miRNAs at baseMean>1. Pink = upregulated in first trimester at
612 FDR<0.05. Purple = upregulated in third trimester at FDR<0.05. Blue = similarly expressed with

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613 P≥0.05. Point labels are the miRNA names minus the "hsa-miR-" prefix. (A) C14MC miRNAs.
614 (B) C19MC miRNAs. (C) Pathway enrichment analysis with experimentally confirmed targets of
615 the S=similarly expressed or D=differentially expressed miRNAs in (i) C14MC or (ii) C19MC.
616

617 **Supplemental Information**

618 **Supplemental File 1. Principal Components Analysis, fetal sex**. PCA plot for the miRNA-seq
619 results of N=113 first trimester and N=47 third trimester placenta samples. The samples cluster
620 by trimester. Samples are color-coded by trimester group and fetal sex, female (F) and male (M).
621 Three patients with matched samples are labeled. [PDF]

622 **Supplemental File 2. Principal Components Analysis, race and ethnicity.** PCA plots for the
623 miRNA-seq results of N=113 first trimester (CVS) and N=47 third trimester (PL) placenta
624 samples. Samples shapes indicate race and ethnicity. [PDF]

625 **Supplemental File 3. Analysis of differential miRNA expression between first and third**
626 **trimester human placentae.** Tables of mature miRNAs DESeq2 results annotated with
627 precursor and chromosome information. (A) Mature miRNAs with no duplicate rows. (B) Rows
628 split by the chromosome column, for scatter plots. (C) Rows split by both the chromosome and
629 precursor miRNA columns, for bar plots to count all genomic locations. (D) DESeq2 results with
630 normalized counts for each sample. [Excel .xlsx]

631 **Supplemental File 4.** Compilation of full target gene enrichment analysis results from IPA Core
632 Analysis. [Excel .xlsx]

633 **Supplemental File 5.** Extended pathway enrichment analysis heatmaps for (i) only
634 experimentally confirmed miRNA targets or (ii) both experimentally confirmed and high
635 confidence predicted miRNA targets. (A) Highly expressed miRNAs with baseMean>10,000 in
636 first (pink, "1") or third (purple, "3") trimester. (B, C, D) Similarly (blue, "S") and differentially
637 (green, "D") expressed miRNAs encoded by (B) all chromosomes, (C) C14MC, (D) C19MC.
638 Heatmap data are -$\log_{10}$(P) output from IPA Core Analysis. [PDF]

639 **Supplemental File 6.** Subset of Supplemental File 3 spreadsheets with placenta-specific miRNA
640 clusters, C14MC and C19MC. [Excel .xlsx]

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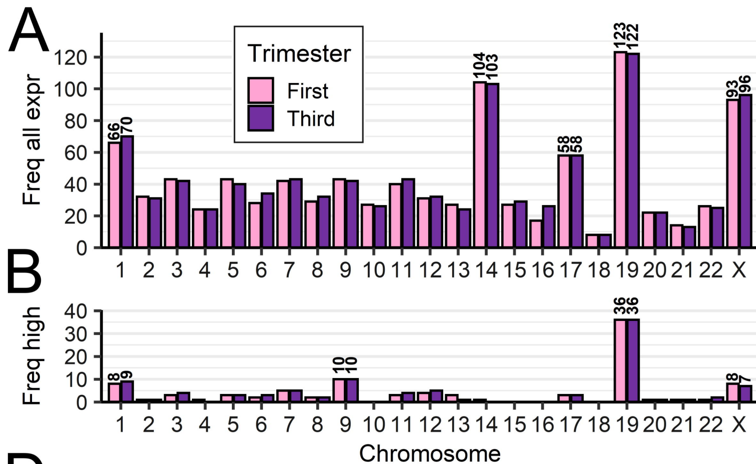
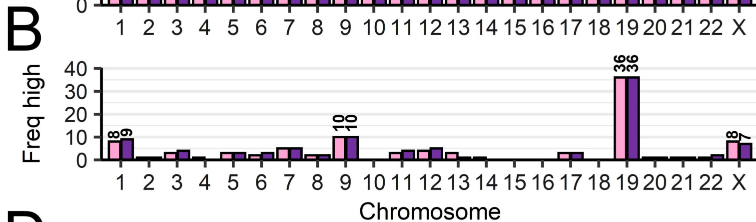
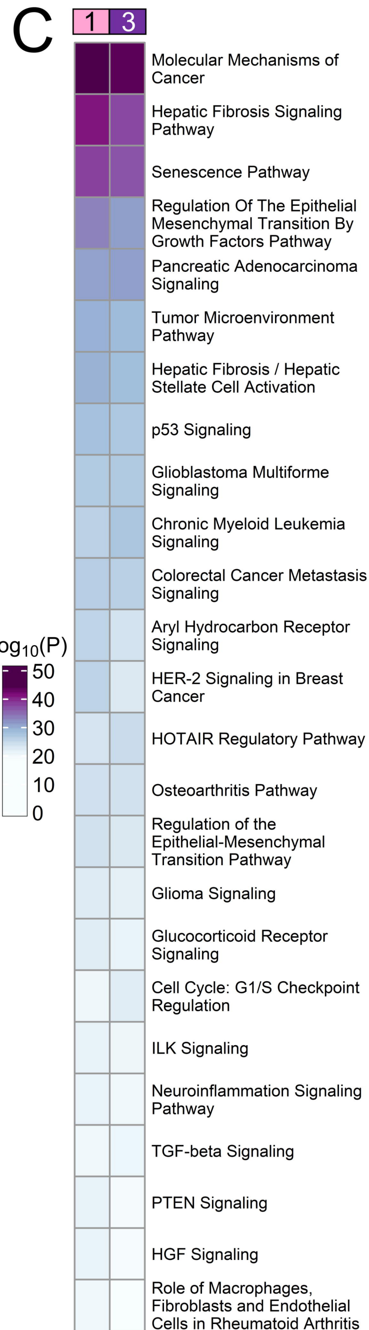
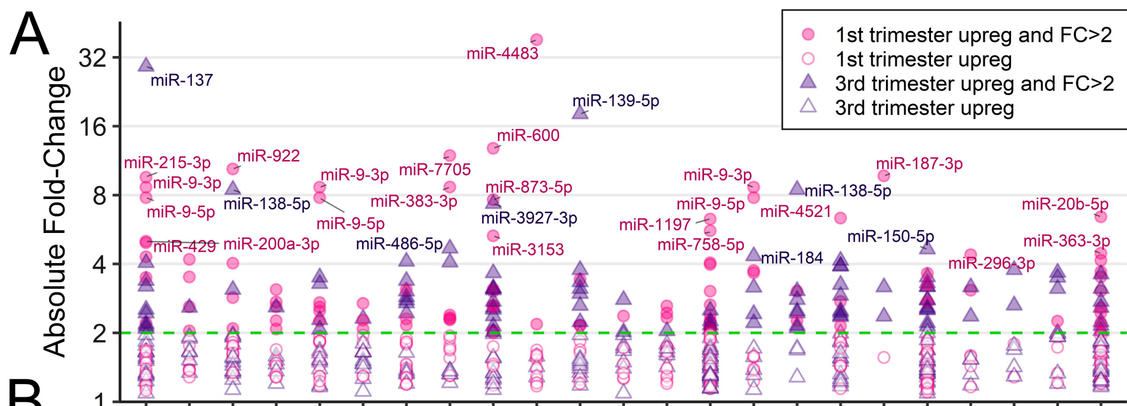


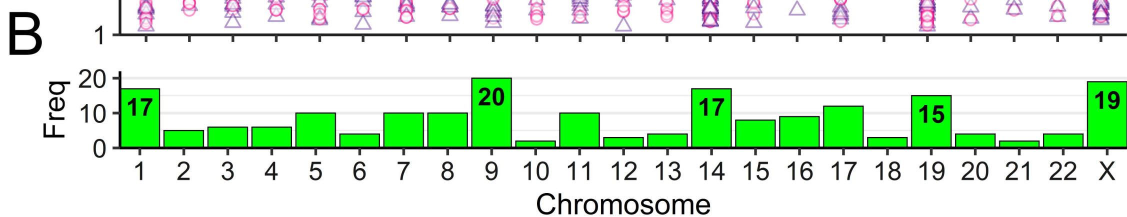


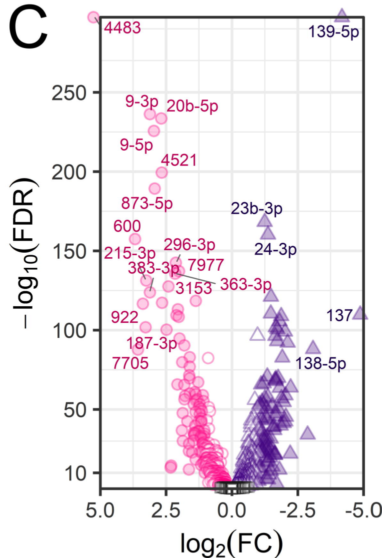


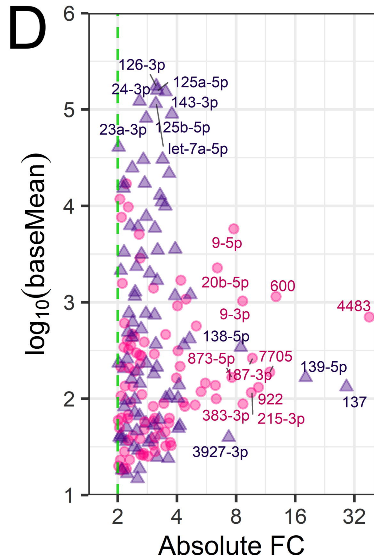
D
Expression: $\log_{10}$(baseMean)
miR-515-5p
miR-518b
miR-1323
miR-518f-3p
miR-335-5p
miR-191-5p
miR-520h
miR-1283
miR-29c-3p
miR-30e-5p
miR-130a-3p
miR-127-3p
miR-515-3p
miR-524-5p
miR-519c-5p
miR-224-5p
miR-223-3p
miR-542-3p
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X

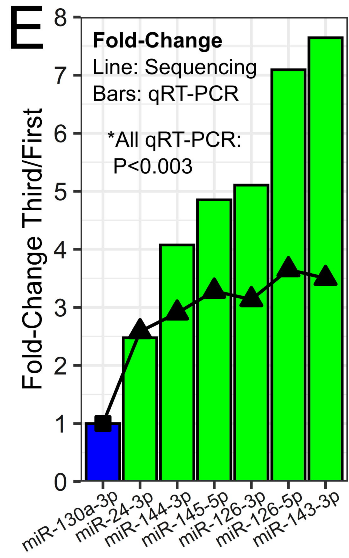
E
Freq
15 21 14 35 20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X
Chromosome

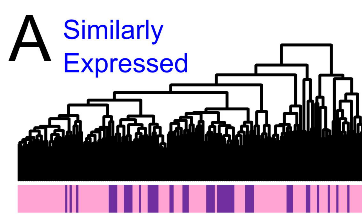

A
Absolute Fold-Change
32
16
8
4
2
1
1st trimester upreg and FC>2
1st trimester upreg
3rd trimester upreg and FC>2
3rd trimester upreg
miR-137
miR-4483
miR-139-5p
miR-600
miR-215-3p
miR-922
miR-9-3p
miR-9-5p
miR-138-5p
miR-7705
miR-383-3p
miR-873-5p
miR-3927-3p
miR-429
miR-200a-3p
miR-486-5p
miR-3153
miR-1197
miR-758-5p
miR-4521
miR-187-3p
miR-20b-5p
miR-150-5p
miR-184
miR-296-3p
miR-363-3p

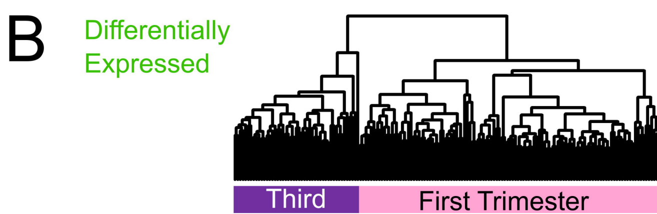

B
Freq
20
10
0
17
20
17
15
19
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X
Chromosome

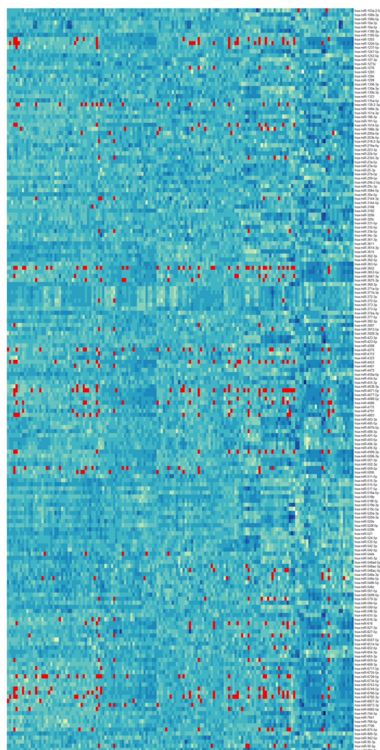

C
$-\log_{10}(\text{FDR})$
$\log_2(\text{FC})$
5.0 2.5 0.0 -2.5 -5.0
10 50 100 150 200 250
4483
139-5p
9-3p
20b-5p
9-5p
4521
873-5p
23b-3p
600
24-3p
215-3p
296-3p
383-3p
7977
3153
363-3p
922
137
187-3p
7705
138-5p

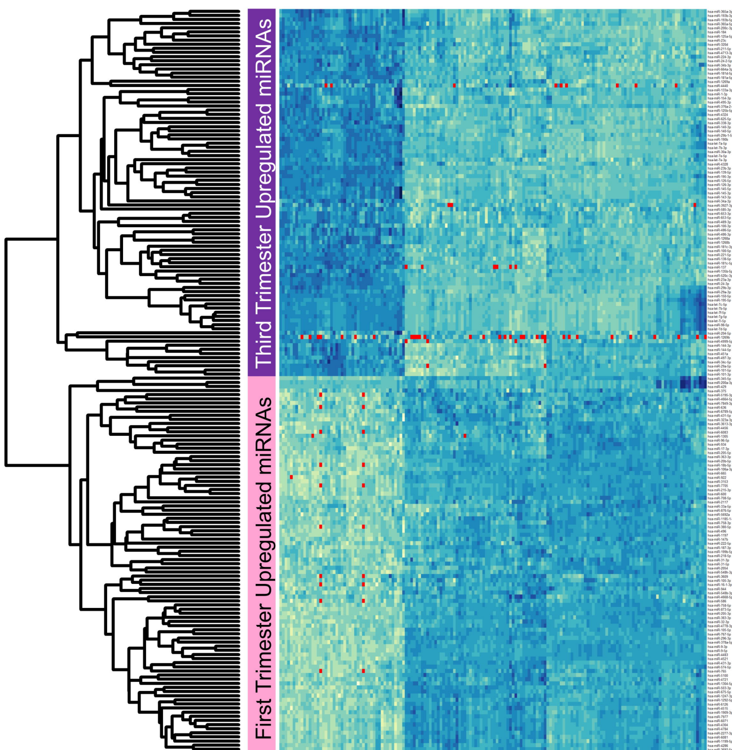

D
$\log_{10}(\text{baseMean})$
Absolute FC
2 4 8 16 32
1 2 3 4 5 6
126-3p
125a-5p
24-3p
143-3p
23a-3p
125b-5p
let-7a-5p
9-5p
20b-5p
600
4483
9-3p
138-5p
873-5p
7705
187-3p
139-5p
922
137
383-3p
215-3p
3927-3p

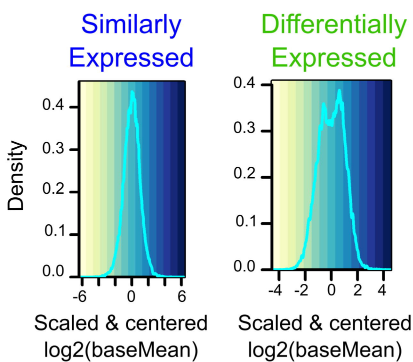

E
Fold-Change Third/First
Fold-Change
Line: Sequencing
Bars: qRT-PCR
*All qRT-PCR: P<0.003
0 1 2 3 4 5 6 7 8
miR-130a-3p
miR-24-3p
miR-144-3p
miR-145-5p
miR-126-3p
miR-126-5p
miR-143-3p

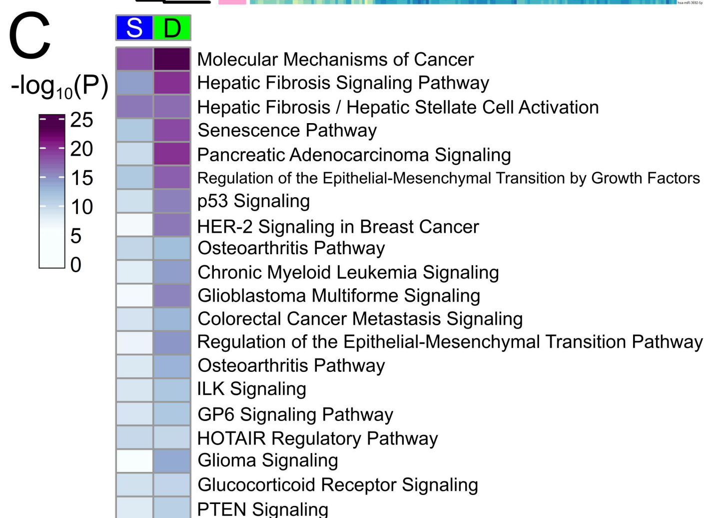

A
Similarly
Expressed

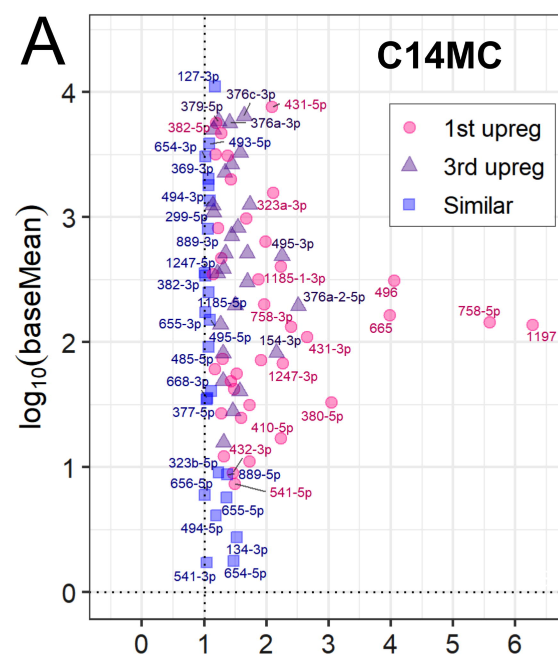

B
Differentially
Expressed
Third
First Trimester




Third Trimester Upregulated miRNAs
First Trimester Upregulated miRNAs


Similarly
Expressed
Differentially
Expressed
Density
Scaled & centered
log2(baseMean)
Scaled & centered
log2(baseMean)
= Not expressed (bM=0)


C
S
D
-log10(P)
25
20
15
10
5
0
Molecular Mechanisms of Cancer
Hepatic Fibrosis Signaling Pathway
Hepatic Fibrosis / Hepatic Stellate Cell Activation
Senescence Pathway
Pancreatic Adenocarcinoma Signaling
Regulation of the Epithelial-Mesenchymal Transition by Growth Factors
p53 Signaling
HER-2 Signaling in Breast Cancer
Osteoarthritis Pathway
Chronic Myeloid Leukemia Signaling
Glioblastoma Multiforme Signaling
Colorectal Cancer Metastasis Signaling
Regulation of the Epithelial-Mesenchymal Transition Pathway
Osteoarthritis Pathway
ILK Signaling
GP6 Signaling Pathway
HOTAIR Regulatory Pathway
Glioma Signaling
Glucocorticoid Receptor Signaling
PTEN Signaling


A
C14MC
1st upreg
3rd upreg
Similar
log10(baseMean)
127-3p
376c-3p
379-5p
431-5p
382-5p
376a-3p
654-3p
493-5p
369-3p
494-3p
323a-3p
299-5p
889-3p
495-3p
1247-5p
382-3p
1185-1-3p
496
1185-5p
376a-2-5p
758-5p
758-3p
665
1197
655-3p
495-5p
154-3p
431-3p
485-5p
1247-3p
668-3p
380-5p
377-5p
410-5p
432-3p
323b-5p
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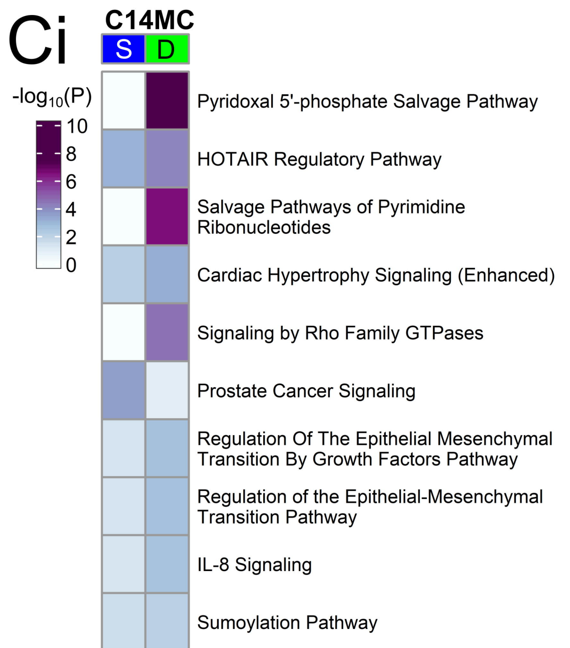
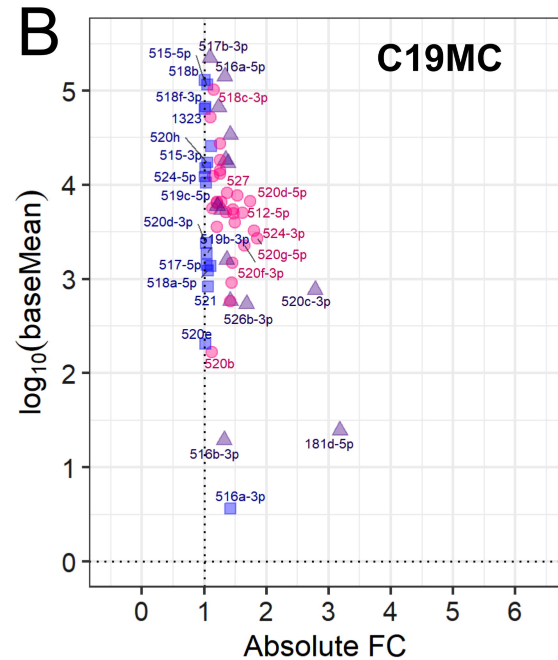
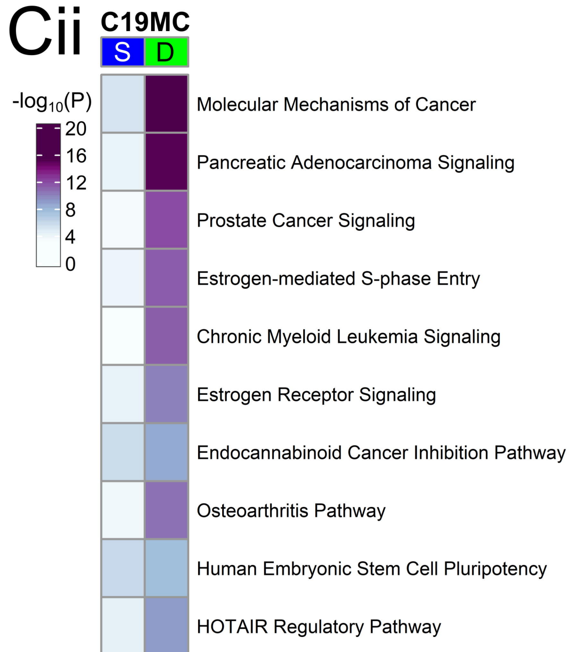
134-3p
541-3p
654-5p

Ci
C14MC
S
D
-log10(P)
Pyridoxal 5'-phosphate Salvage Pathway
HOTAIR Regulatory Pathway
Salvage Pathways of Pyrimidine Ribonucleotides
Cardiac Hypertrophy Signaling (Enhanced)
Signaling by Rho Family GTPases
Prostate Cancer Signaling
Regulation Of The Epithelial Mesenchymal Transition By Growth Factors Pathway
Regulation of the Epithelial-Mesenchymal Transition Pathway
IL-8 Signaling
Sumoylation Pathway

B
C19MC
log10(baseMean)
Absolute FC
515-5p
517b-3p
516a-5p
518b
518f-3p
518c-3p
1323
520h
515-3p
524-5p
527
519c-5p
520d-5p
512-5p
520d-3p
524-3p
519b-3p
520g-5p
517-5p
520f-3p
518a-5p
521
520c-3p
526b-3p
520e
520b
516b-3p
181d-5p
516a-3p

Cii
C19MC
S
D
-log10(P)
Molecular Mechanisms of Cancer
Pancreatic Adenocarcinoma Signaling
Prostate Cancer Signaling
Estrogen-mediated S-phase Entry
Chronic Myeloid Leukemia Signaling
Estrogen Receptor Signaling
Endocannabinoid Cancer Inhibition Pathway
Osteoarthritis Pathway
Human Embryonic Stem Cell Pluripotency
HOTAIR Regulatory Pathway