

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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55 Author contributed to conceptualization and design of the work (TLG, LEE, MDP), acquisition  
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57 (TLG, LEE, AEF, DW, YW, JT, JLC), and interpretation of the data (TLG, LEE, AEF, DW,  
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60

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

## 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.<sup>1</sup> 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.<sup>2-5</sup> This  
104 occurs during a relative state of hypoxia, which promotes trophoblast invasion and  
105 angiogenesis.<sup>6-9</sup> 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.<sup>10-12</sup> 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.<sup>13,14</sup>

111 MicroRNAs (miRNAs) are non-coding, single-stranded RNA molecules of about 22 nucleotides  
112 in length.<sup>15</sup> 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.<sup>16,17</sup> While some miRNAs are universally  
115 expressed, others are expressed preferentially or exclusively in certain tissues.<sup>18</sup> Two large  
116 miRNA clusters are enriched in placenta, the chromosome 14 miRNA cluster (C14MC) and the  
117 chromosome 19 miRNA cluster (C19MC).<sup>19,20</sup> C14MC is a large, imprinted, maternally-  
118 expressed miRNA cluster, with several members predominantly expressed in placenta and  
119 epithelial tissues.<sup>18</sup> 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.<sup>18,20-24</sup>

122 Widespread regulation of gene expression by miRNAs, the presence of placental-specific  
123 miRNAs, and miRNA expression differences in various trophoblastic cell lines<sup>25</sup> 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<sup>26-40</sup> and intrauterine growth restriction  
127 (IUGR).<sup>39-43</sup> 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,<sup>44-46</sup> 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.<sup>25,47,48</sup> 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,

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 *RNAlater* 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 *RNAlater* 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.<sup>49,50</sup>  
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.<sup>49,50</sup> 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 *RNAlater* buffer, tissue was sonicated on ice in lysis buffer

184 (600  $\mu$ L RLT Plus lysis buffer with 1%  $\beta$ -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,<sup>51</sup> 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.<sup>52,53</sup> 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).<sup>54</sup> 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



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.<sup>55,56</sup> 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  $P \geq 0.05$ ,  
227 absolute fold-change  $\leq 2$ , and baseMean>10 in both trimesters. The  $P \geq 0.05$  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  $P \geq 0.05$  (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.<sup>49,57,58</sup> 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.<sup>59</sup> 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.

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 $\leq 2$ , 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  $\log_2(\text{baseMean})$  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<sup>-</sup>  
264 <sup>13</sup>) between first and third trimester. A highly expressed miRNA with stable expression in first  
265 and third trimester placenta 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  $\Delta\Delta C_t$  method,<sup>60</sup> with hsa-miR-130a-3p as an internal reference. Statistics were  
271 performed using the Wilcoxon rank-sum test on  $\Delta C_t$  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

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

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  $\leq 2$  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

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=3.69x10<sup>-298</sup>), 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=4.52x10<sup>-97</sup>) 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

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 ( $P \geq 0.05$ ,  $\text{baseMean} > 1$ ), 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 ( $\text{FDR} < 0.05$  and  $\text{baseMean} > 1$ ), 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,  $\text{FDR} = 4.83 \times 10^{-118}$ ), hsa-miR-758-5p (5.59-  
393 fold,  $\text{FDR} = 5.07 \times 10^{-101}$ ), hsa-miR-496 (4.05-fold higher in first,  $\text{FDR} = 3.92 \times 10^{-109}$ ), and hsa-miR-  
394 665 (3.98-fold,  $\text{FDR} = 1.75 \times 10^{-95}$ ), 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,  $\text{FDR} = 2.91 \times 10^{-111}$ ), followed by hsa-miR-181d-5p (3.18-fold higher,  $\text{FDR} = 6.38 \times 10^{-70}$ ),  
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

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.

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.<sup>61,62</sup> 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.<sup>63</sup> Although  
442 variation among the first and second trimester was not different,<sup>63</sup> 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,<sup>64</sup> 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<sup>65</sup>, 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.<sup>66-68</sup> 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.<sup>35,69</sup> It has also been associated with preterm birth<sup>70</sup> and fetal growth restriction.<sup>43</sup>  
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,<sup>71</sup> and may not  
461 be used solely as a biomarker of disease, but may be incorporated with other miRNAs with



462 stable expression across gestation that change with disease using a bivariate biomarker disease  
463 approach described by Laurent.<sup>45</sup>

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.<sup>2</sup> 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.<sup>72</sup>

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.<sup>73,74</sup> MiRNAs have been implicated in the epithelial to mesenchymal transition.<sup>75-80</sup>

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.<sup>75-79</sup> In human trophoblast cell lines it has been identified to silence MED1 under  
489 hypoxic conditions.<sup>75</sup> suggesting it has a role in the first trimester regulating trophoblast  
490 differentiation during physiologic hypoxic conditions.<sup>75,81,82</sup> “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

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,<sup>83</sup> consistent with first trimester placental function when extravillous trophoblasts  
500 degrade and induce ECM remodeling to enable migration.<sup>2,5,84,85</sup>

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, Syncytin-1 (*ERVWE1*), which mediates cell-fusion-induced  
505 senescence of the syncytiotrophoblast.<sup>86-88</sup> 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.<sup>89</sup>

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.<sup>66</sup> Similar to  
515 other studies using whole villous tissue and primary cytotrophoblasts,<sup>20,48,63</sup> 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.<sup>63</sup>

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

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).<sup>25,47,48,90</sup> 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

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.

579 **Figure/Table Legends**

580 Table 1 - Demographics

	<b>First Trimester</b>	<b>Third Trimester</b>	<b>P value</b>
<b>N</b>	110	44	
<b>Maternal age, years</b>	37.7 (3.0)	37.3 (3.0)	0.65
<b>Paternal age, years</b>	39.5 (4.8)	38.6 (4.8)	0.30
<b>Maternal race/ethnicity</b>			
Caucasian	106 (96.4%)	35 (79.6%)	0.002
Non-Hispanic	107 (97.3%)	35 (79.6%)	<0.001
<b>Paternal race/ethnicity</b>			
Caucasian	104 (94.6%)	37 (84.1%)	0.04
Non-Hispanic	107 (97.3%)	36 (81.8%)	0.001
<b>Fetal race/ethnicity</b>			
Caucasian	103 (93.6%)	32 (72.7%)	<0.001
Non-Hispanic	106 (96.4%)	33 (75.0%)	<0.001
<b>Fetal female sex</b>	57 (51.8%)	18 (40.9%)	0.22
<b>Maternal pre-pregnancy BMI, kg/m<sup>2</sup></b>	21.9 (3.4)	24.0 (4.7)	0.005
<b>Maternal pre-existing medical conditions</b>			
Hypertension	0 (0%)	0 (0%)	-
Diabetes	0 (0%)	0 (0%)	-
Thyroid disorder	3 (2.7%)	6 (13.6%)	0.02
<b>Pregnancy complications</b>			
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%)	-
<b>Hypertension management in pregnancy</b>			
Anti-Hypertensives	0 (0%)	3 (6.8%)	0.022
Any Magnesium use (Ante- or Postpartum)	0 (0%)	6 (13.6%)	<0.001
<b>Mode of delivery- Cesarean section</b>	33 (30%)	15 (34.1%)	0.74
<b>Gestational age at delivery, days</b>	276.3 (7.0)	276.5 (8.0)	0.43
<b>Birthweight, g</b>	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.

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  $\text{baseMean} > 10$  or (B) the most highly expressed with  $\text{baseMean} > 10,000$ . (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  $P \geq 0.05$  and  $FC \leq 2$ . The red line ( $\text{baseMean} = 10$ ) is the threshold selected for stable  
591 expression. (E) Counts of similarly expressed miRNAs with  $P \geq 0.05$ ,  $FC \leq 2$ ,  $\text{baseMean} > 10$  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  $\text{baseMean} > 10$ . The dotted line represents  $FC = 2$ . (B)  
596 Chromosome frequency of 180 DE miRNA precursors at  $FDR < 0.05$ ,  $FC > 2$ ,  $\text{baseMean} > 10$ . (C)  
597 Volcano plot of all miRNAs with  $\text{baseMean} > 10$ . Key as in A, with addition of open black  
598 squares for non-significant miRNAs ( $FDR \geq 0.05$ ). (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  $\log_2(\text{baseMean})$ , columns = hierarchically clustered samples.  $\text{BaseMean} = 0$   
605 samples are highlighted red. (A) 182 similarly expressed miRNAs with  $P \geq 0.05$ ,  $FC \leq 2$ , and  
606  $\text{baseMean} > 10$ . The miRNAs are listed alphabetically. (B) 180 differentially expressed miRNAs  
607 with  $FDR < 0.05$ ,  $FC > 2$ , and  $\text{baseMean} > 10$ . 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  $\text{baseMean} > 1$ . Pink = upregulated in first trimester at  
612  $FDR < 0.05$ . Purple = upregulated in third trimester at  $FDR < 0.05$ . Blue = similarly expressed with

613  $P \geq 0.05$ . 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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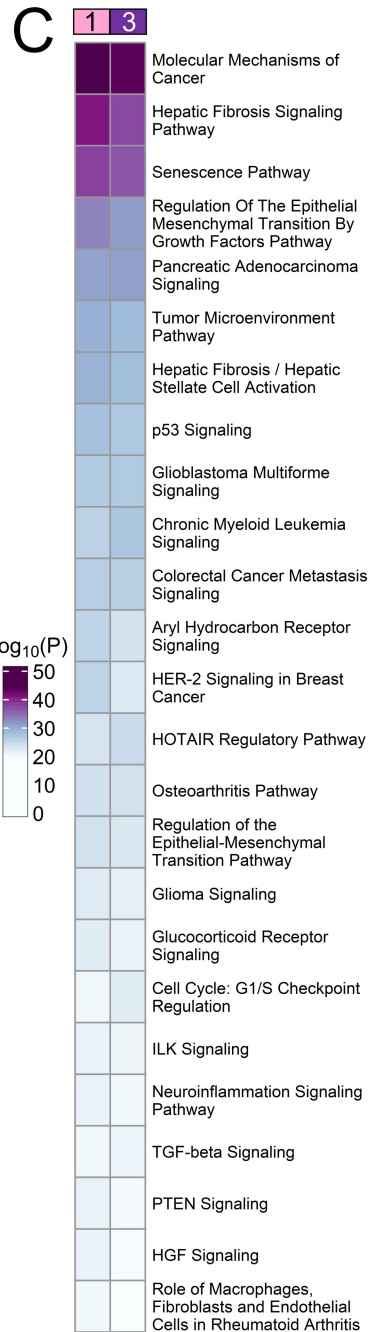
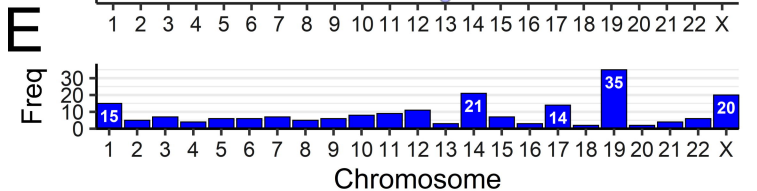
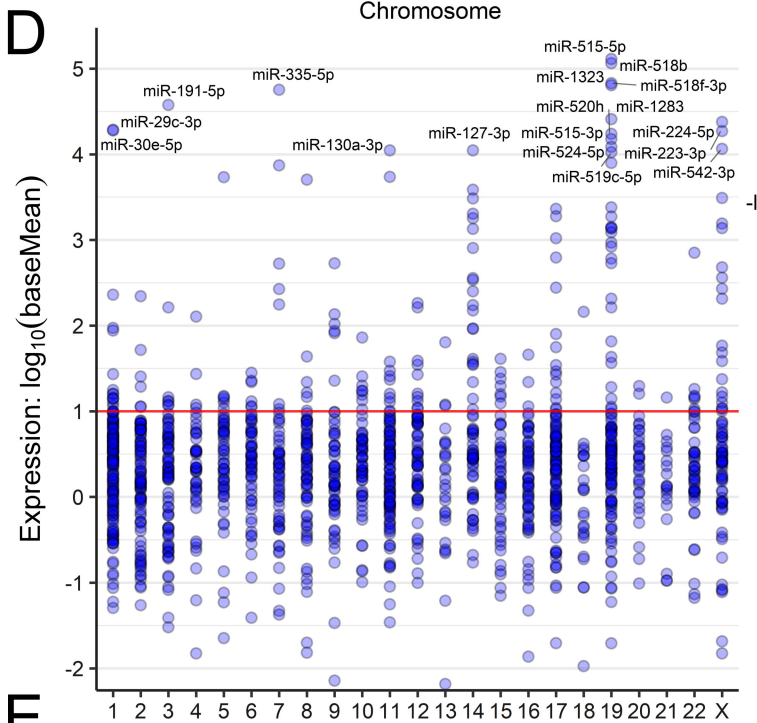
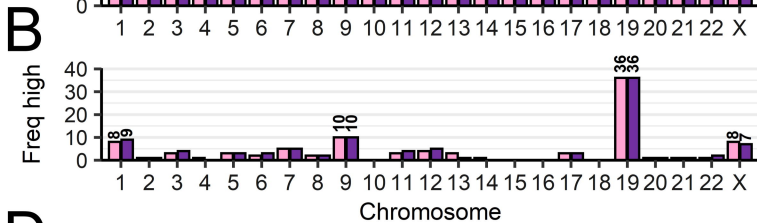
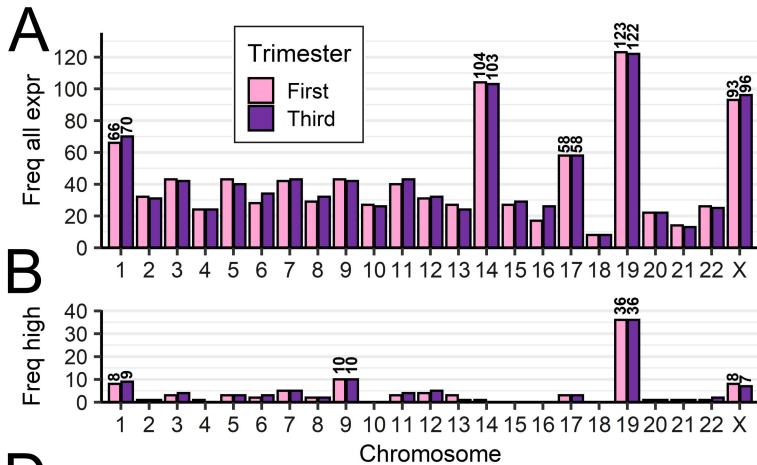
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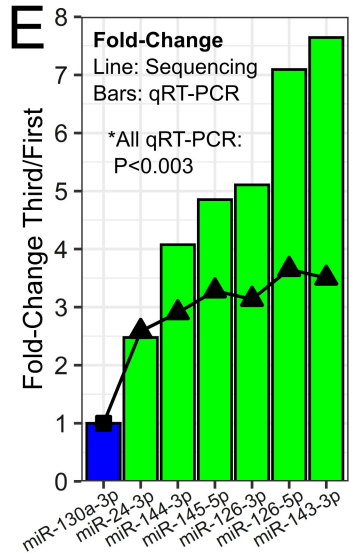
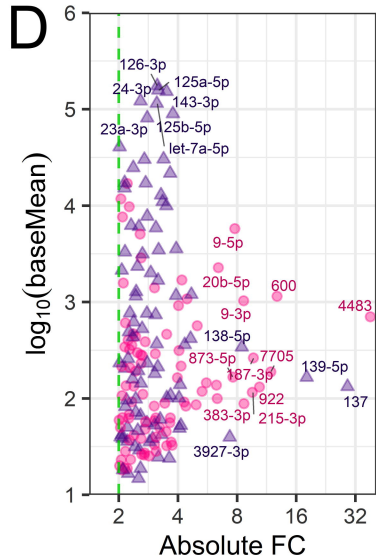
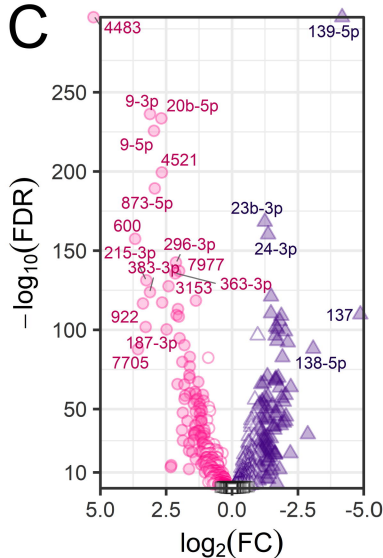
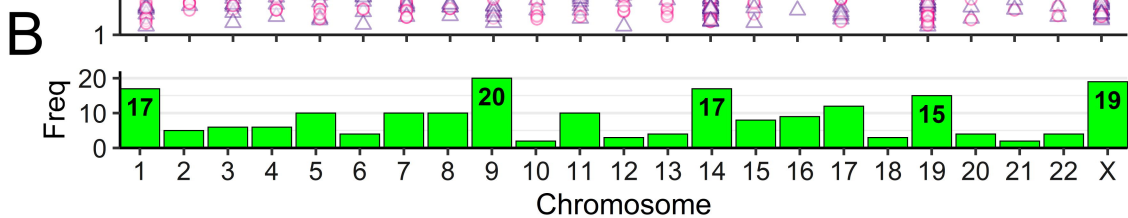
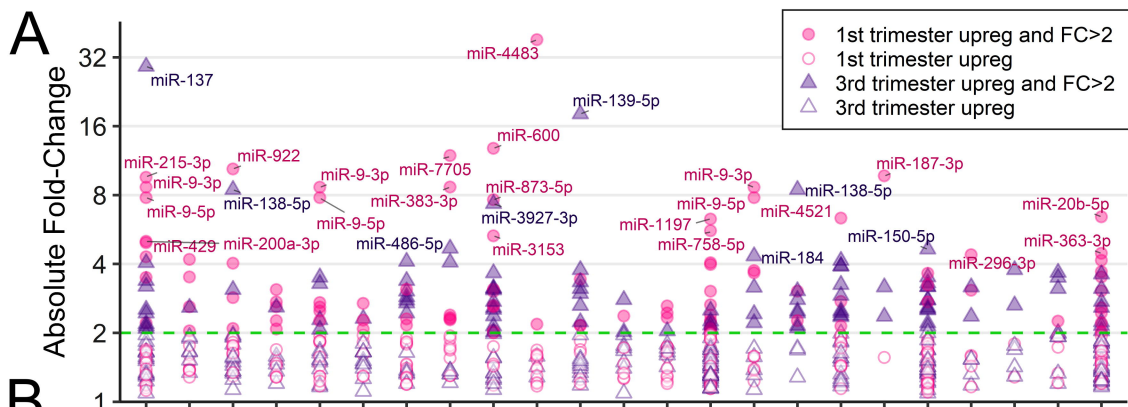
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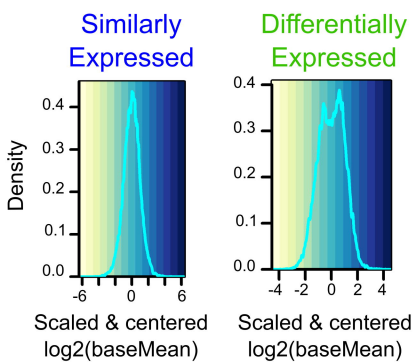
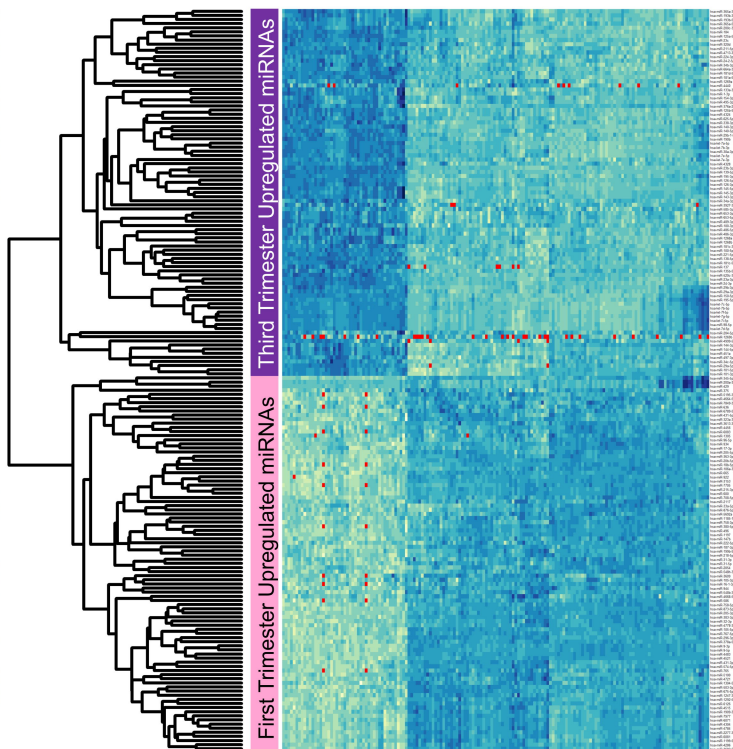
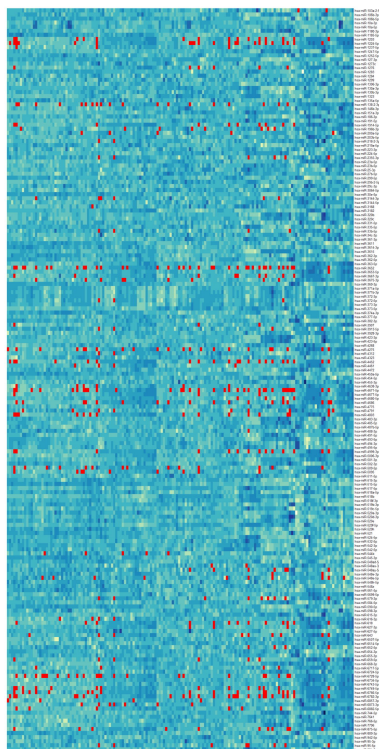
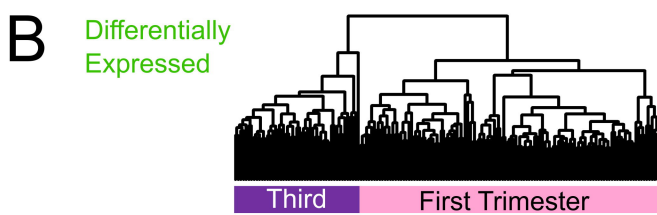
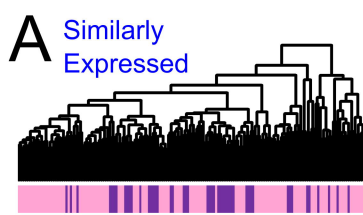
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■ = Not expressed (bM=0)

