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Large-scale transcriptome-wide profiling of microRNAs in human placenta and maternal plasma at early to mid gestation

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ABSTRACT

MicroRNAs (miRNAs) are increasingly seen as important regulators of placental development and opportunistic biomarker targets. Given the difficulty in obtaining samples from early gestation and subsequent paucity of the same, investigation of the role of miRNAs in early gestation human placenta has been limited. To address this, we generated miRNA profiles using 96 placentas from presumed normal pregnancies, across early gestation, in combination with matched profiles from maternal plasma. Placenta samples range from 6 to 23 weeks' gestation, a time period that includes placenta from the early, relatively low but physiological (6–10 weeks' gestation) oxygen environment, and later, physiologically normal oxygen environment (11–23 weeks' gestation).

We identified 637 miRNAs with expression in 86 samples (after removing poor quality samples), showing a clear gestational age gradient from 6 to 23 weeks' gestation. We identified 374 differentially expressed (DE) miRNAs between placentas from 6–10 weeks' versus 11–23 weeks' gestation. We see a clear gestational age group bias in miRNA clusters C19MC, C14MC, miR-17 ~ 92 and paralogs, regions that also include many DE miRNAs. Proportional change in expression of placenta-specific miRNA clusters was reflected in maternal plasma.

The presumed introduction of oxygenated maternal blood into the placenta (between ~10 and 12 weeks' gestation) changes the miRNA profile of the chorionic villus, particularly in placenta-specific miRNA clusters. Data presented here comprise a clinically important reference set for studying early placenta development and may underpin the generation of minimally invasive methods for monitoring placental health.

Introduction

The human placenta is a complex, rapidly developing and highly regulated organ shared between two genetically unique individuals, the mother and her foetus. Its function is vital for the transfer of nutrients, gases and wastes between maternal and foetal circulations, with dysfunction leading to placental insufficiencies and pregnancy complications [1,2]. Essential placental functions are mediated by the syncytiotrophoblast, large multinucleated cells covering the chorionic villi and providing a large surface area for bi-directional exchange of molecules between foetal and maternal circulations.

Early development of the human placenta occurs in a relatively low, but physiological, oxygen environment due to the occluding presence of extravillous cytotrophoblasts (EVTs) in the uterine spiral arterioles. The placenta transitions to that which is usually considered a physiologically normal, oxygen environment over several weeks from 10 weeks' gestation when EVTs become dislodged permitting maternal blood to flow into the placental intervillous space

[1,3]. This period of rising oxygen tension is a critical time for placental development, as an appropriate response to the accompanying burst of oxidative stress is crucial to the ongoing success of the pregnancy [3].

Robust characterization of important regulatory mechanisms surrounding normal placental development can give insight into pregnancy health, and the potential development of pregnancy complications [2,4]. Identification of biomarkers of ectopic oxygenation or placental dysfunction leading to pregnancy complications is an important step in monitoring pregnancy health.

MicroRNAs (miRNAs) are an important class of RNA molecules that can help us to understand regulation of developmental pathways within human tissues. As such they are prime targets for biomarker research. miRNAs are short, approximately 20–24 nucleotide (nt) lengths of single-stranded, non-coding ribonucleic acid (RNA) originating in non-coding regions of the genome [5] and comprehensively reviewed in Bartel (2018) [6]. They most commonly function

as post-transcriptional regulators of gene expression either by translational repression or targeted degradation by cleavage of mRNA transcripts in the cytoplasm [7,8]. miRNAs target mRNA regulatory sequences that are often located in the 3′ untranslated region (UTR) of the mRNA transcript with potential for multiple conserved target sites within a single mRNA [5,9]. Approximately half of known mammalian miRNAs are found within intergenic regions of the genome, with the remaining miRNAs mostly transcribed from the open reading frame of other genes, including long non-coding RNA (lncRNA) [10–12]. Adding to this complexity, miRNA may be transcribed as a single, or polycistronic unit [11].

The critical role of the placenta in successful pregnancy is clear, and there is also evidence linking developmental programming of chronic adult disease such as heart disease, diabetes and obesity with the placental phenotype *in utero* [4,13,14]. However, there are currently no non-invasive tests to determine which women are likely to develop pregnancy complications in routine use, nor is there a comprehensive reference set of miRNA expression during early pregnancy that can be used for development of pregnancy health biomarkers. In this study, we performed high-throughput sequencing to generate robust miRNA expression profiles for 96 placentas and matched maternal plasma samples between 6–23 weeks' gestation, creating a comprehensive set of miRNA expression profiles for presumably healthy placental function. We identified differential miRNA expression between 6–10 and 11–23 weeks' gestation which could reflect physiological changes occurring in this important time period. Using placenta-associated miRNA clusters found on chromosome 14, 19, 13, 7 and X, we also investigated how maternal plasma reflects placental profiles to enable future biomarker discoveries.

Methods

Sample collection

Placental chorionic villous tissue samples were obtained with informed, written consent from women undergoing elective terminations of otherwise healthy pregnancies. Samples of peripheral blood (6–9 mL) were collected from the same women into standard EDTA blood tubes at the time of termination and stored on ice until processed. Whole blood underwent centrifugation at 800 x g for 15 min at 4°C before plasma removal and then spun for a further 15 min. All samples were stored at −80°C until further processing. Prior to termination, accurate gestational age was determined using transvaginal ultrasonography. Termination samples were collected from the Pregnancy Advisory Centre (PAC), Woodville, South Australia. All placenta samples were assessed for gross morphology before inclusion in the study.

RNA extraction and library preparation

All placenta tissues were collected post termination and processed as soon as possible after collection and placed into RNALater (Thermo Fisher) within 15 min. Total RNA was isolated using a modified protocol of the RNeasy mini plus kit (Qiagen, Hilden, Germany) whereby the RW1 buffer, a proprietary component of the RNeasy Kit that eliminates small RNAs, is replaced with 100% ethanol. The sample is then subjected to size fractionation for small RNAs before sequencing. For plasma, miRNA was isolated from 200 μL plasma samples using the QIAGEN miRNA serum/plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were stored at −80°C until further processing. miRNA real-time qPCR for the assessment of haemolysis was conducted by QIAGEN Genomic Services (Qiagen, Hilden, Germany). 2 μl RNA was reverse transcribed in 10 μL reactions using miRCURY LNA Kit (QIAGEN version5). Each RT was performed including an artificial RNA spike-in (UniSp6). cDNA was diluted 50 x and assayed in 10 μl PCR reactions according to the protocol for miRCURY LNA miRNA PCR; each miRNA was assayed once by qPCR using assays for miR-23a, miR-30 c, miR-103, miR-142-3p, and miR-451. In addition to these miRNA assays, the RNA spike-ins were assayed. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analysed using in-house software, both for the determination of the Cq (by the 2nd derivative method) and for melting curve analysis.

All placenta samples were genotyped for foetal sex using high resolution melt curve analysis of the gene that encodes amelogenin. Amelogenin is found on both the X (AMELX) and Y (AMELY) chromosomes, the X allele features a 3 bp deletion in exon 3 allowing the identification of samples with only X chromosomes (female) or X and Y chromosomes (male) prior to sequencing. Forward primer 5′-CCCTGGGCTCTGTAAAGAATAGTG-3′, reverse primer 5′-ATCAGAGCTTAAACTGGGAAGCTG-3. qPCR was performed using SSOFast EvaGreen Supermix (Bio-Rad, CA), primers at 250 nM final concentration, 5 ng of DNA per reaction, on a Bio-Rad CFX384 Real-Time PCR System. Cycling conditions: initial denaturation 98°C 30s, 40 cycles of 98°C for 5s and 60°C for 5s. High resolution melt curve analysis was performed from 65°C to 85°C with a 0.2°C increment every 10s. Melt curve between 65°C and 70°C was analysed using Bio-Rad Precision melt software (Bio-Rad, CA) to identify sex genotypes. Library preparation and sequencing was performed by Qiagen (Valencia, CA) using the QIAseq miRNA Library Kit and QIAseq miRNA 48 Index IL kits as per manufacturer's instructions. Amplified cDNA libraries underwent single-end sequencing by synthesis (Illumina 1.9).

High-throughput sequencing analysis

Alignment and analysis of miRNA sequencing data were processed from raw FASTQ files using the *bcbio-nextgen* pipeline [15]. Briefly, adapter detection and trimming were performed using Atropos [16]. Alignment was performed using STAR [17] using the human reference genome build GRCh37[18],(https://www.ncbi.nlm.nih.gov/grc. Quality control metrics were assessed using FastQC[19] (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to check for per base sequence quality, sequence length distribution and

duplication levels, and summarized using multiQC [20]. miRNA were clustered and collapsed using SeqCluster [21], and individual miRNAs detected using SeqBuster [15]. Annotation was performed using the miraligner [15] with miRBase version 21.0 [22,23] (http://www.mirbase.org) as the reference database.

Differential expression analysis

All profile and expression analyses were conducted in the R statistical environment (v.3.3.2), using the *edgeR* (v.3.16.5) and *limma* [24] (v.3.30.11) R/Bioconductor packages. *edgeR* [25] was used to filter miRNA with low expression and normalize for library composition bias. Batch effects, introduced by sequencing samples in multiple sequencing runs, were evident after normalization and corrected using a *limma* batch correction method [24]. All samples were then normalized using the Trimmed Mean of M values (TMM). For plasma, $\log_2$ transformed counts per million (CPM) were 1 to 1 paired with placenta data prior to creation of plasma figures. Briefly, after creating design matrices for both wanted (gestational age and tissue type) and unwanted (sample independent) effects, the *limma* method remove Batch Effects was employed to regress out the effect of patient derived variation from the matched plasma count.

Sample-weights and log transformation was performed using *limma* package [26] with the voom function used to estimate the mean-variance relationship between individual observations and then applied to the normalized log-count data. Differential expression analysis including, moderated F-statistic evaluation, adjusted p-value estimation and Log Fold Change (LFC) analysis were performed using a moderated t-test [27] with Benjamini-Hochberg (BH) multiple hypothesis test corrections [28]. The data were first pooled per gestational age group and then compared. After adjustment, expression of miRNAs was considered significantly different at FDR ≤ 0.05. The workflow, source code and input files associated with this research available at (https://github.com/mxhp75/earlyPlacentamiRNA_SeqProfile.git).

Results

Characterizing a comprehensive miRNA profile of chorionic villous tissue across early gestation to characterize the human placental miRNA profile across early to mid gestation, Illumina NextSeq 75 bp single-end read sequencing was performed on miRNA libraries from chorionic villous samples obtained from 96 singleton pregnancies following elective pregnancy terminations (44 females and 52 males bearing pregnancies). An average of 18.72 million reads were sequenced per sample (range ~10–29 million reads; Additional file 1: Table S1). miRNA counts were generated by mapping to human miRBase V21 [22]. Libraries with low sequencing depth were removed, including samples PAC0131 (14,595 reads) and PAC0071 (removed due to low mapping after initial read count of 1,819,847 reads). Subsequent unsupervised clustering (Additional file 2: Figure S1) using principal component analysis (PCA) showed eight additional samples (PAC0041, PAC0039, PAC0045, PAC0008,

PAC0035, PAC0024, PAC0034, PAC0006) that segregated from the remaining 86 samples, most likely due to the presence of adjacent non placental villous tissue. This hypothesis was tested using matched DNA methylation profiles which confirmed the presence of decidual tissue [29], leading to the removal of these samples from subsequent analyses.

After filtering, miRNAs were aligned to the human GRCh37 reference genome and counts produced from 4,665 miRBase annotations [22], identifying an initial set of 2,032 expressed miRNAs. To remove sequencing noise, miRNAs with <5 reads across all samples were removed. A total of 1,422 miRNAs were identified with non-zero expression means across all 86 samples, representing a standard known miRBase miRNA expression set for early to mid gestation human placenta. After accounting for sequencing batch effects, very low abundance miRNAs were discarded by converting to $\log_2$ CPM values and removing miRNAs with expression <2.5 CPM in ≥29 samples (see methods), leaving a total of 637 robust miRNAs for downstream profile analyses. An additional 588 candidate *de novo* miRNA sequences were predicted through miRDeep2 analysis.

Of the 637 identified miRNAs, the top ten miRNAs with the highest mean expression across all samples in placenta were miR-30d-5p, miR-125a-5p, miR-517a-3p, miR-199a-3p, miR-26b-5p, miR-26a-5p, let-7a-5p, miR-21-5p, miR-126-3p and miR-516b-5p (Table 1a). For a full list of miRNAs identified and profiled in this study see Additional File 1: Table S2. A principal component analysis (PCA) of all 637 miRNAs (Figure 1) indicates a clear gradient across early gestation. The PCA identified 23.2% variance accounted for in PC1, with the top 10 miRNAs contributing to PCA dimension one (PC1) being: miR-519 c-3p, miR-328-3p, miR-519b-3p, miR-20a-5p, miR-19b-3p, miR-501-3p, miR-485-5p, miR-3605-3p and miR-515-5p, miR-106a-5p (Additional file 1: Table S3). Interestingly both −3p and −5p arms of the miR-20a transcript, previously associated with placental angiogenesis [30], contributed highly to the gestational age change associated with PC1.

Differentially expressed miRNAs across early gestation

Given the importance of oxygen tension in orchestrating the growth and differentiation of the placenta between 6 and 23 weeks' gestation, we separated the 86 samples in our cohort by their gestation relative to the presumed introduction of oxygenated maternal blood flow into the intervillous space. Using prior knowledge we designated that ≤10 weeks' (6–10 weeks') gestation be considered pre- and >10 weeks' (11–23 weeks') gestation be considered post-initiation of maternal blood flow into the intervillous space [3]. This enabled us to identify changes in miRNA expression across this time-point, and to independently assess highly expressed miRNA in each group. When miRNAs from all 86 samples were analysed according to expression prior to and post 10 weeks' gestation, after accounting for potentially confounding factors such as maternal age, maternal BMI, maternal smoking status, we identified 13 constitutively expressed miRNAs with consistent high expression ($\log_2$ CPM 14.35–15.60) in both gestation groups (Table 1b). Notably, 4 of the top 20 most highly
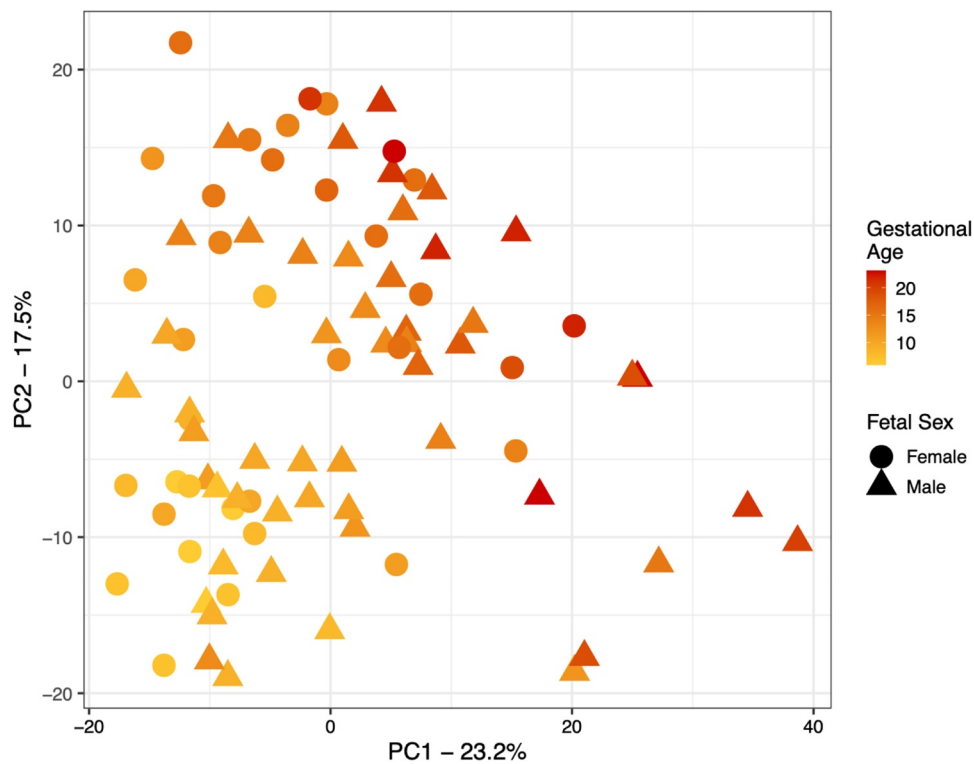
Figure 1. Unsupervised clustering of placental chorionic villous samples after batch correction. The PCA plot indicates a clear gestational age gradient from early (left) to later gestation (right) in the first dimension. This trend reflects the gestational age signature identified in our miRNA expression data.

Table 1. miRNA with highest expression in early gestation placenta. Global miRNA expression was calculated using the average expression across early gestation (6–23 weeks') and $\log_2$ transformed. (**a**) Top ten miRNA with highest average global expression. (**b**) Constitutively highly expressed miRNA with membership in the top ten by highest average expression if calculated independently for the 6–10 weeks' and 11–23 weeks' gestation groups.

a				b		
miRNA	miRNA Family	Avg. Global Expression ($\log_2$ CPM)		miRNA	miRNA Family	Avg. Expression ($\log_2$ CPM)
						6–10 weeks' / 11–23 weeks'
miR-30d-5p	miR-30-5p	15.60				
miR-125a-5p	miR-125-5p	15.51		miR-30d-5p	miR-30d-5p	15.53 / 15.64
miR-517a-3p	miR-517-5p	15.45		miR-125a-5p	miR-125-5p	14.93 / 15.74
miR-199a-3p	miR-199-3p	15.43		miR-517a-3p	miR-517-5p	15.52 / 15.40
miR-26b-5p	miR-26-5p	15.25		miR-199a-3p	miR-199-3p	15.21 / 15.56
miR-26a-5p	miR-26-5p	14.84		miR-26b-5p	miR-26-5p^	15.27 / 15.21
let-7a-5p	let-7-5p/98-5p	14.47		let-7a-5p	let-7-5p/98-5p	13.59 / 14.83
miR-21-5p	miR-21-5p/590-5p	14.39		miR-21-5p	miR-21-5p/590-5p	14.20 / 14.59
miR-126-3p	None listed	14.31		miR-126-3p	None listed	13.91 / 14.56
miR-516b-5p	miR-516b-5p	14.30		miR-516b-5p	miR-516b-5p	13.23 / 14.31
				miR-16-5p	miR-15-5p/16-5p/195-5p/424-5p/497-5p	14.34 / 14.32
				miR-516a-5p	miR-516a-5p	14.40 / 14.11
				miR-143-3p	miR-143-3p	13.60 / 14.52
				miR-26a-5p	miR-26-5p^	14.54 / 14.99
					^shared seed region	

expressed miRNAs are from the placenta-specific chromosome 19 cluster, previously identified as being highly expressed in placenta [31–33].

After identifying the highly expressed miRNAs in placenta, we then investigated the change in expression that occurs after 10 weeks' gestation once maternal blood flow into the placenta is initiated and presumably when oxygen tension begins to rise. Differential expression analysis identified 374 significantly (FDR <0.05) different miRNAs, with 163 down-regulated and 211 up-regulated in the 11–23 weeks' gestation placenta compared to 6–10 weeks' gestation (Figure 2a; Additional file 1: Table S4). Strongly downregulated

miRNAs (negative $\log_2$FC) include miR-4483, miR-129-5p, miR-124-3p and miR-122-5p, and highest up-regulated (positive $\log_2$FC) include miR-4645-3p, miR-195-3p, miR-137, miR-139-3p, miR-6715b-3p, miR-3927-3p and miR-1269b. Differentially expressed miRNAs had predominantly low expression (Figure 2b) with an average $\log_2$ expression >10 only observed in miR-9-5p ($\log_2$FC of −1.43, Figure 2c) and let-7a-5p ($\log_2$FC of 1.22, Figure 2d). We see that for both let-7 and miR-9, the 5′ and 3′ arms follow a similar expression trajectory. Further examples are provided in Additional file 2, Figure S2. These mature fragments were originally transcribed as a single transcript before further processing (Figure 2e).
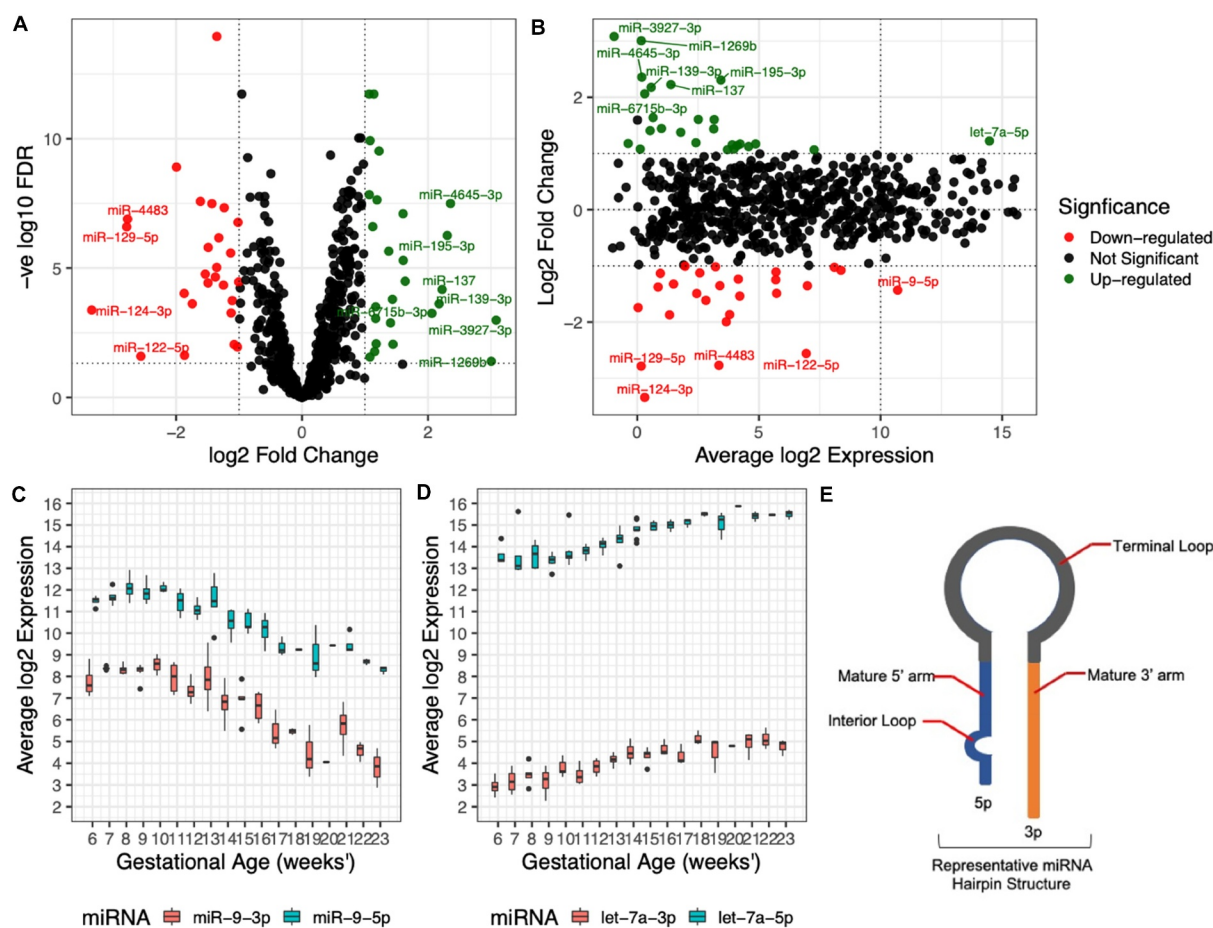
Figure 2. Differential expression analysis between 6–10 weeks' and 11–23 weeks' gestation placenta. (a) Volcano plot of differential expression. Linear regression identified 26 down-regulated with a $\log_2$FC <-1 (red) and 26 up-regulated with a $\log_2$FC >1 (green) miRNA between gestation groups. (b) MA plot of $\log_2$ fold change as a function of $\log_2$ average expression. Box plots of average expression as a function of gestational age for miR-9-5p/3p (c) and let-7a-5p/3p (d) show a clear changing pattern of expression from early to mid gestation. (e) Schematic of a representative miRNA hairpin structure indicates the origin of the 5′ and 3′ arms of the mature miRNA transcript.

Conserved placenta-associated miRNA clusters display variable expression between early and mid gestation

In vertebrate species miRNAs are significantly enriched in clusters, with ~50% of miRNA clusters being the result of random or non-local duplications. The placenta-associated clusters analysed herein included chromosome 19 miRNA cluster commonly known as C19MC (19q13.41) [34], chromosome 14 cluster (C14MC; 14q.32) [35], and chromosome 13 cluster (miR-17 ~ 92; 13q31.3) with its paralogs the chromosome X cluster miR-106a~363 (Xq26.2), and chromosome 7 cluster miR-106b~25 (7q22.1) [36]. We identified the expression of 48 mature miRNAs from C19MC, 77 from C14MC and 20 from miR-17 ~ 92 and paralogs. Of the 48 mature miRNAs identified in the C19MC cluster, 34 miRNA had significant (FDR <0.05) differential expression, that were down regulated in 11–23 weeks' compared to 6–10 weeks' gestation placenta (Additional file 1: Table S5). C19MC cluster expression, as a proportion of total miRNA transcripts, was reduced after 10 weeks' gestation (Figure 3a). Interestingly, while miRNAs in this cluster have been demonstrated to be transcribed by RNA-Pol II as a single polycistron [37], the individual members display a range of expression levels (Figure 3b).

The C14MC cluster, that contains 77 mature miRNAs, had 56 miRNAs with significant (FDR <0.05) differential expression. 55 of these miRNAs were up-regulated, while only one miRNA (miR-410-5p; FDR 1.99×10^{-3}) was down-regulated in the 11–23 weeks' gestation group (Additional file 1: Table S6). C14MC-member transcript expression, as a proportion of total expression, increased across early to mid gestation in placenta, further differing from C19MC finding by being more centred around the group mean (Figure 4a). Similar to C19MC cluster members, we found a range of expression levels amongst the C14MC members (Figure 4b) that are also thought to be transcribed as a single polycistronic transcript, suggesting more complex regulation of primary miRNA transcripts processing [38]. The miRNA miR-412-5p, previously observed as highly expressed in first trimester placenta [39], is notable in that it appears to have a bimodal distribution of expression (Figure 4c) that, upon further investigation, was not found to be related to sample characteristics such as foetal sex, maternal age, maternal BMI or maternal smoking status.

Of the 20 mature miRNAs in the miR-17 ~ 92 cluster and its paralogs, 17 had significant (FDR <0.05) differential
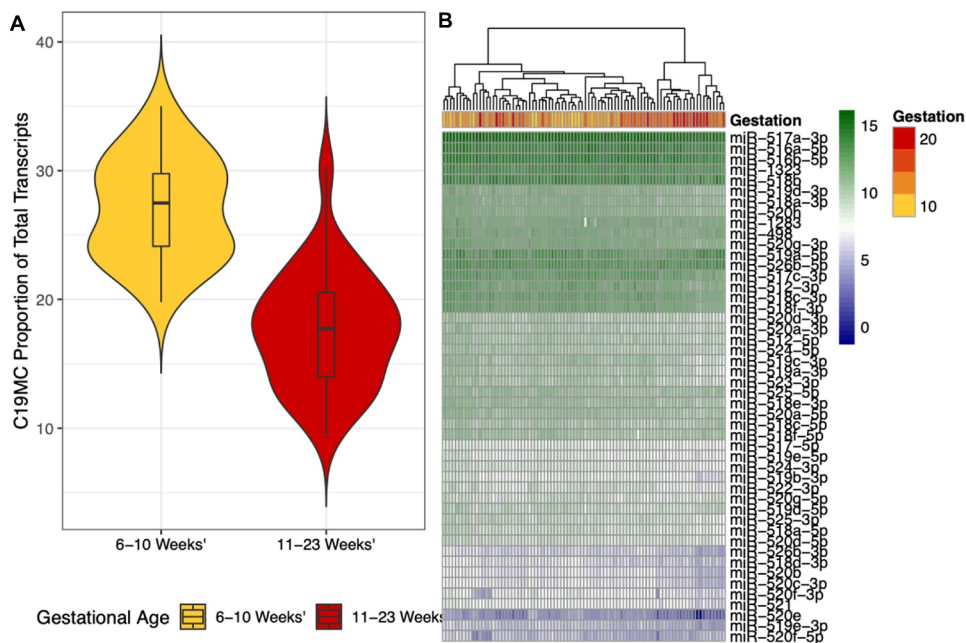
Figure 3. The proportion of C19MC transcripts decreased after 10 weeks' gestation in placenta. The proportion of combined C19MC member expression as a function of total miRNA expression is decreased in 11–23 weeks' compared to 6–10 weeks' gestation (violin plot) (a) with expression of individual cluster members tending to decrease across early to mid gestation (b).
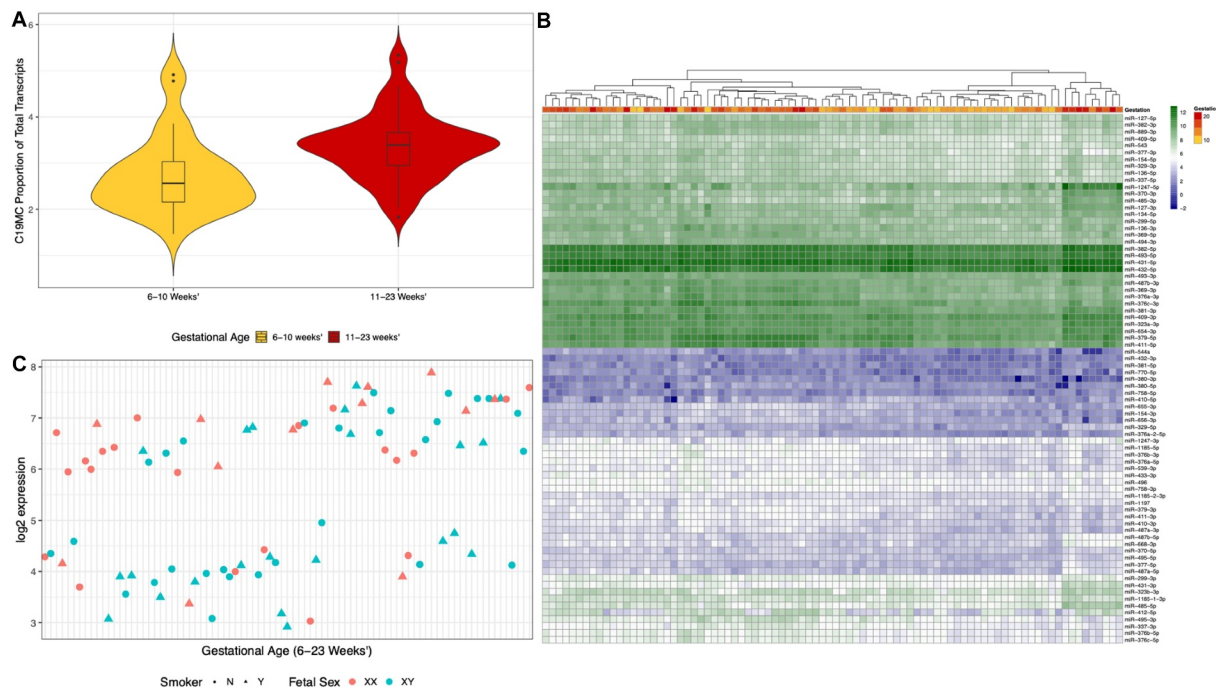
Figure 4. The proportion of C14MC transcripts increased in 11–23 weeks' compared to 6–10 weeks' gestation placenta. The proportion of C14MC member expression as a function of total miRNA expression is increased after 10 weeks' gestation compared to 6–10 weeks' gestation (violin plot) (a). Individual cluster members' expression is increased across early gestation in 55 of the 56 miRNAs (b). miR-412-5p appears to have a bimodal distribution which is unexplained by foetal sex, maternal age or maternal smoking status (c).

expression with 15 being down- and 2 being up-regulated in the 11–23 weeks' gestation group compared to the 6–10 weeks' (Additional file 1: Table S7). Investigation into the proportion of placenta expression originating from the miR-17 ~ 92 cluster and its paralogs revealed a pattern similar to that of the C19MC member transcripts with the proportion of transcripts identified in placenta tissue as a function of total transcripts decreasing across early gestation (Figure 5(a–c)). We identified a variation in expression patterns between each of the three paralogs (Figure 5d), with chromosome 13 expression decreasing across early to mid gestation.

Whilst we find significant (FDR <0.05) differential expression in all of the above miRNA clusters, the log$_2$FC between 6–10 weeks' and 11–23 weeks' gestation groups is small (C19MC log$_2$FC −1.02 to −0.19; C14MC log$_2$FC 0.23 to 0.94 and log$_2$FC 0.67; miR-17 ~ 92 log$_2$FC −0.2 to −1.13 and log$_2$FC 0.84 to 0.89), with changes in the proportional expression of each cluster as a function of total miRNA expression suggesting an important regulatory mechanism for these clusters in placenta.

Characterizing a comprehensive profile of placental and endogenous miRNA in maternal plasma across early gestation

To characterize the miRNA profile across early to mid gestation in maternal plasma, we sequenced 96 1 to 1 matched maternal plasma samples (hereafter referred to as plasma) from women sampled at the time of pregnancy termination using the same sequencing protocol as detailed for the chorionic villous tissue. Plasma and tissue samples from each termination are assigned a common unique identifier, with a suffix used to separate sample types. miRNA sequencing resulted in approximately 17.77 million 75 bp single-end reads per sample in plasma (range 9,986,347–41,344,361 reads: Additional file 3: Table S8) across two sequencing runs. After initial quality control and sequence analysis, we identified a number of samples with aberrant expression of miR-451a and miR-16 indicating potential for red blood cell miRNA contamination. We performed a subsequent analysis using the delta-delta CT method (ΔΔCt) to calculate the relative fold change of gene expression of samples using quantitative polymerase chain reaction (qPCR). The ΔΔCt value of miR-23a minus miR-451 is examined, as miR-23a is known to be stable in samples affected by haemolysis whilst miR-451 is known to vary with contamination. This method identified 11 samples (PAC0033, PAC0034, PAC0041, PAC0048, PAC0050, PAC0051, PAC0054, PAC0056, PAC0062, PAC0084 and PAC0105) as more likely to contain haemolysis contamination, a common issue in plasma research. Despite potential issues involving haemolysis, we aimed to establish the presence of placenta-specific miRNAs within maternal plasma by including all available samples regardless of whether they had matched placenta data or not.

Alignment and filtering were performed using the same protocol as for chorionic villous tissue. The number of unique miRNA species identified per sample varied, ranging from 1022 miRNA species in PAC0077 to 355 miRNA species in PAC0010. Prior to filtering we identified 1775 unique mature miRNAs across all plasma samples, with a core set of 790 high confidence miRNAs present after filtering. Of these, 531 were also present in placenta samples, 259 unique to plasma, and 106 miRNAs unique to placenta (Additional file 3: Table S9). Unsupervised clustering (PCA) of all 790 high confidence miRNAs identified in plasma shows no gestational age gradient for maternal plasma from 6 to 23 weeks' gestation (Additional file 2: Figure S3).

Given the variability of miRNA in plasma, we hypothesized that the detection of placental miRNAs in plasma may be restricted to miRNA present in high concentrations in matched placenta. We therefore investigated whether 10 miRNAs (Table 1a) with the highest abundance in placenta were present in plasma and found that all ten miRNA were at high abundance levels in matched plasma (Figure 6, Table 2a). Many of the most highly expressed plasma miRNA are red blood cell (RBC) associated.

Of the 790 miRNAs identified in plasma, the top ten miRNAs with the highest global expression were miR-16-5p, miR-486-5p, miR-92a-3p, let-7a-5p, let-7b-5p, miR-451a, let-7 f-5p, miR-25-3p, let-7i-5p, miR-93-5p (Table 2b). These were also all highly expressed in placental tissue (average global expression of 8.95–14.47 Log$_2$ normalized CPM). miR-141, miR-200b, miR-139 and miR-184, previously identified in third trimester plasma, were also identified at high abundance in our early gestation plasma, with miR-141-3p abundance increasing across early gestation, as shown in previous studies [40].

We investigated plasma levels of the 21 miRNAs identified as both differentially and highly expressed in the matched placentas (Table 3). Whilst all 21 placental miRNAs were also detected in plasma, their origin is not necessarily placenta-specific, with cellular enrichment linked to various systemic compartments [41]. For example, five of these miRNAs have cellular enrichment in immune response, another three miRNAs in pluripotent and embryonic stem cells, and one miRNA is enriched in extra-embryonic stem cells. The latter may originate from the foetal membranes or other extra-embryonic tissue such as chorionic villi.

We further investigated the abundance of plasma miRNAs from three miRNA clusters previously identified as highly placenta-specific or placenta associated [34–36]. We found 44 mature miRNA from the C19MC cluster, 48 miRNA from the C14MC cluster and 20 miRNA from the miR-17 ~ 92 cluster and its paralogs in plasma. These cluster miRNAs have previously been the target of biomarker research in pregnancy health but to our knowledge these are not being used clinically.

The change in relative proportion of expression by miRNA cluster members in plasma across the 10–11 weeks' gestation threshold is difficult to detect with C14MC and C19MC members each representing less than 1% of the miRNA abundance quantified in plasma. As such the proportional change identified in placenta is not entirely reflected in plasma. Abundance levels of the miR-17 ~ 92 cluster and its paralogs represent approximately 0.03% of the total miRNA identified in plasma.
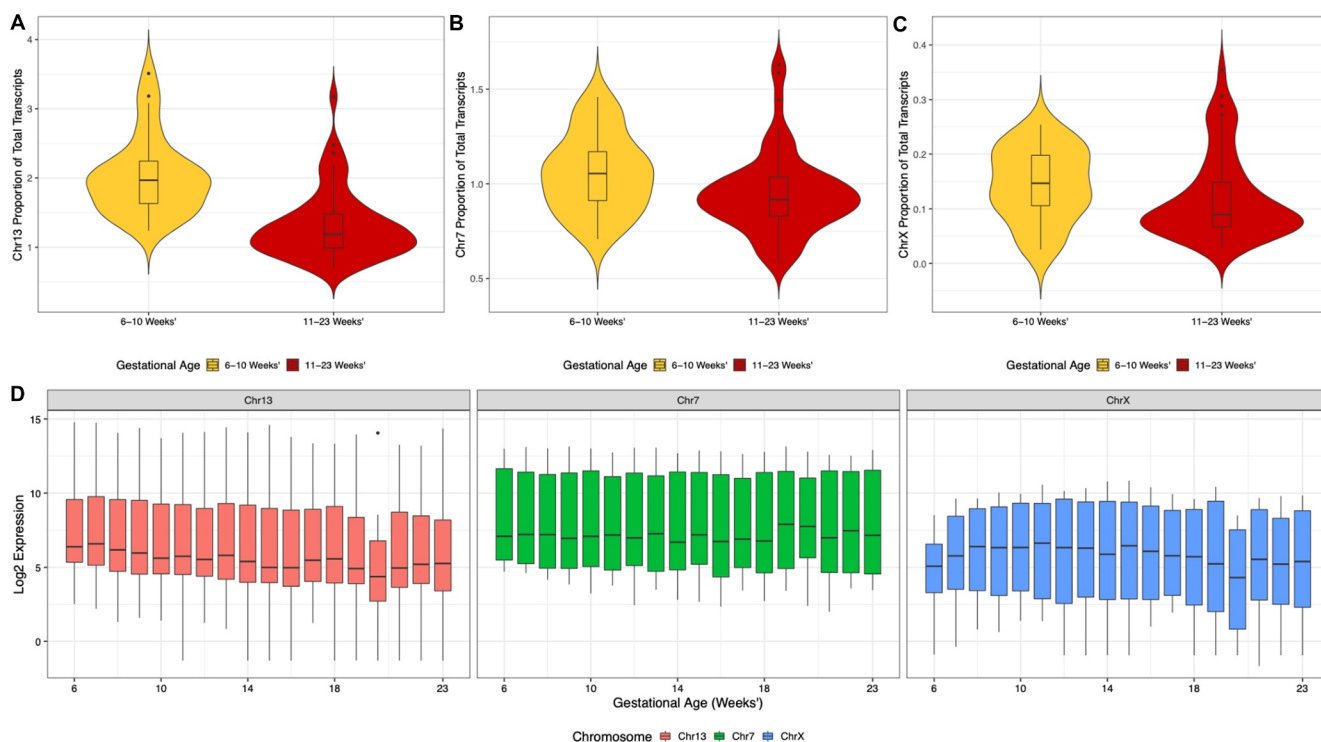
Figure 5. The proportion of miR-17 ~ 92 (Chr 13) and paralogs 106b~25 (Chr 7) and 106a~363 (Chr X) transcripts decreased in 11–23 weeks' compared to 6–10 weeks' gestation placenta. Violin plot showing decreased proportion of miR-17 ~ 92 member expression as a function of total miRNA expression after 10 weeks' gestation (a, b, c) with a general trend of decreased expression of individual cluster members across early gestation (d). Although chromosome 13 and chromosome X appear correlated, expression of miR-17 ~ 92 (chromosome 13) and its paralogs miR-106a~363 (chromosome 7) and miR-106b~25 (X chromosome) does not appear to follow a common expression pattern in placental tissue as we would expect if all 3 clusters are identically regulated by C-Myc.

Discussion

In this study, we have assessed the miRNA profile of 96 human placenta and matched plasma samples in early to mid gestation (6–23 weeks'), generating a placental tissue dataset using high-throughput sequencing that is substantially larger than previous reports that used microarray and qPCR profiling. We derive robust and comprehensive profiles that define miRNA expression at high-resolution, applying stringent bioinformatic protocols for the analysis of sequencing data, akin to large atlas studies.

Differentially expressed miRNAs reflect the dynamically changing utero-placental environment during early to mid gestation

In order to determine the potential association between placental miRNA expression and pregnancy health, particularly the expression of highly abundant miRNAs across early gestation, we examined more closely a period in early gestation involving a steep rise in oxygen tension in the intervillous space (IVS). Rising oxygen tension occurs between 10 and 12 weeks' gestation, at the onset of maternal blood flow into the IVS, resulting in an oxidative burst in placenta [3]. Previous studies have addressed miRNA response to hypoxia and the introduction of oxygen [42–46], including how ectopic oxygen tension may contribute to diseases of pregnancy [42,47–49]; and reviewed in [50]. The number and extensive gestation range of our samples enabled us to analyse the

differential expression of miRNAs prior to, during and post the critical 10–11 weeks' gestational time point. This has potential to lead to identification of placental mechanisms for the remediation of oxidative stress at this crucial time in pregnancy.

That the placenta develops initially in a condition of low (but physiological) oxygen tension has been known for some time [3], and it has been suggested that this is important for the protection of the early foetus from the effects of oxidative stress such as tissue damage from oxygen free radicals [51]. miRNAs are uniquely poised to play a significant role in the mediation of gene expression throughout the early change of oxygen tension in the placenta by employing post transcriptional regulatory mechanisms to rapidly and precisely fine tune the placental environment in response to oxidative stress. We identified upregulation of let-7b/e-5p, miR-21, miR-23a/b-3p, miR-24-3p and miR-199a-5p, also associated with *HIF1a*, in the 6–10 weeks' gestation group. All of these have previously been shown to be upregulated by hypoxia. We also found lower abundance of let-7a and miR-101-3p in our 6–10 weeks' gestation group, both of which have previously been shown to be downregulated by hypoxia [52–56]; and reviewed in [57]. miRNAs identified in this study likely play an important role in the mitigation and regulation of hypoxia in the placenta. This may prove important to our understanding of diseases of pregnancy such as preeclampsia and early pregnancy loss.

The importance of miRNA function in the early placenta is not limited to the mitigation of oxidative stress but also in

Table 2. miRNA by average expression in maternal plasma. Average miRNA expression was calculated using the average normalized CPM expression across early to mid gestation (6–23 weeks') and log$_2$ transformed. **(a)** All top 10 miRNA with highest abundance in placenta were also detected at high abundance in plasma. Two miRNA (miR-517a-3p and miR-516b-5p) originate from the C19MC. **(b)** Top 10 miRNA by abundance in maternal plasma. RBC-associated miRNA identified herein have been previously identified in plasma samples including adult plasma and umbilical cord plasma but have not previously been profiled extensively in maternal plasma in early to mid gestation.

a			b		
miRNA	miRNA Family	Avg. Global Expression (log$_2$ CPM)	miRNA	miRNA Family	Avg. Global Expression (log$_2$ CPM)
miR-30d-5p	miR-30-5p	13.35	miR-16-5p[+]	miR-15-5p/16-5p/195-5p/424-5p/497-5p	18.23
miR-125a-5p	miR-125-5p	10.59	miR-486-5p[+]	miR-486-5p	17.46
miR-517a-3p^	miR-517-5p	4.71	miR-92a-3p[+]	miR-25-3p/32-5p/92-3p/363-3p/367-3p	16.22
miR-199a-3p	miR-199-3p	10.62	let-7a-5p	let-7-5p/98-5p	15.70
miR-26b-5p	miR-26-5p	12.84	let-7b-5p	let-7-5p/98-5p	15.55
miR-26a-5p	miR-26-5p	12.70	miR-451a[+]	miR-451	14.42
let-7a-5p	let-7-5p/98-5p	15.70	let-7 f-5p	let-7-5p/98-5p	14.15
miR-21-5p	miR-21-5p/590-5p	12.87	miR-25-3p	miR-25-3p/32-5p/92-3p/363-3p/367-3p	13.97
miR-126-3p	None listed	13.41	let-7i-5p	let-7-5p/98-5p	13.92
miR-516b-5p^	miR-516b-5p	4.27	miR-93-5p	miR-17-5p/20-5p/93-5p/106-5p/519-3p	13.58

^ C19MC   [+] RBC

Table 3. The abundance in plasma of miRNA differentially expressed in matched placenta. Average miRNA expression was calculated using the average CPM expression across early to mid gestation (6–23 weeks') and log$_2$ transformed.

Placenta			Maternal Plasma		
Placenta DE miRNA	log$_2$FC	AveExp (log$_2$)	AveExp (log$_2$)	Compartment of origin*	Cellular enrichment^
let-7a-5p	1.24	14.27	15.70	brain, epididymis, spinal cord	Mesodermal cells
miR-542-5p	1.11	7.07	0.66	epididymis, small intestine, lymph node	None listed
miR-222-3p	1.00	9.66	6.51	bladder, epididymis, prostate	Endothelial cell, endothelial cell of vascular tree, blood vessel endothelial
miR-1-3p	1.00	7.20	4.45	muscle, myocardium	Muscle, skeletal muscle, mesodermal cell
miR-199b-5p	0.97	10.24	2.72	colon, epididymis, oesophagus, skin, thyroid, vein	Fibroblast, mesodermal cell
miR-99a-5p	0.98	8.57	5.92	Arachnoid mater, brain, epididymis, prostate, spinal cord	Mast cell, leukocyte, myeloid leukocyte
miR-223-3p	0.99	10.93	13.28	vein	Leukocyte, haematopoietic cell, myeloid leukocyte
miR-221-3p	0.94	13.13	9.65	Epididymis, prostate	Endothelial cell, ciliated epithelial cell, muscle cell
miR-100-5p	0.98	12.83	5.58	Arachnoid mater, nerve, spinal cord, brain	Muscle cell, smooth muscle cell, vascular associated smooth muscle cell
miR-181a-5p	0.94	13.06	10.09	Arachnoid mater, brain, dura mater, spinal cord	Leukocyte, haematopoietic cell, B cell
miR-363-3p	0.94	9.18	9.26	Epididymis, vein	Leukocyte, haematopoietic cell, lymphocyte
miR-122-5p	−2.53	6.74	13.09	Liver, vein	Hepatocyte, endodermal cell
miR-9-5p	−1.42	10.51	2.19	Brain, spinal cord	Neural cell, neuroectodermal cell, ectodermal cell
miR-9-3p	−1.37	6.79	−2.25	Brain, dura mater, spinal cord	None listed
miR-372-3p	−1.08	8.17	−2.98	No profile	Pluripotent stem cell, embryonic stem cell, induced pluripotent stem cell, depleted in extraembryonic stem cell
miR-371a-5p	−1.01	7.90	−2.81	Constitutive expression	Pluripotent stem cell, embryonic stem cell, induced pluripotent stem cell, depleted in extraembryonic stem cell
miR-520 f-3p	−0.98	6.86	−3.07	spleen	Pluripotent stem cell, embryonic stem cell, induced pluripotent stem cell, depleted in extraembryonic stem cell
miR-934	−0.93	9.32	−2.44	Constitutive expression, highest in gallbladder and pleura	Epithelial cell, extraembryonic cell, endo-epithelial cell
miR-20a-5p	−0.85	10.02	9.73	Constitutive expression, highest in vein	Pluripotent stem cell, epithelial cell, endothelial cell
miR-17-5p	−0.77	6.80	8.77	Constitutive expression, highest in vein	Epithelial cell, pluripotent stem cell, endothelial cell
miR-373-3p	−0.68	6.74	−2.57	kidney	Pluripotent stem cell, embryonic stem cell, induced pluripotent stem cell

* [83]
^ [84]

cellular proliferation, trophoblast invasion and cellular differentiation, which are important to the placenta and also to the success of the pregnancy. let-7a-5p expression in first trimester placental explants has been implicated in a reduction in cytotrophoblast proliferation. In our differential expression analysis we identified let-7a-5p as up-regulated in the 11–23 weeks' gestation compared to the 6–10 weeks' gestation group. Our detailed week-by-week expression data suggests an incremental increase across early to mid gestation which together with previous term data is consistent with let-7a-5p's proposed role in regulation of placental growth by the reduction of cytotrophoblast proliferation [58]. The miR-34 family is known as a tumour suppressor miRNA family due to their synergistic effects with p53 [59]. Consistent with this, we
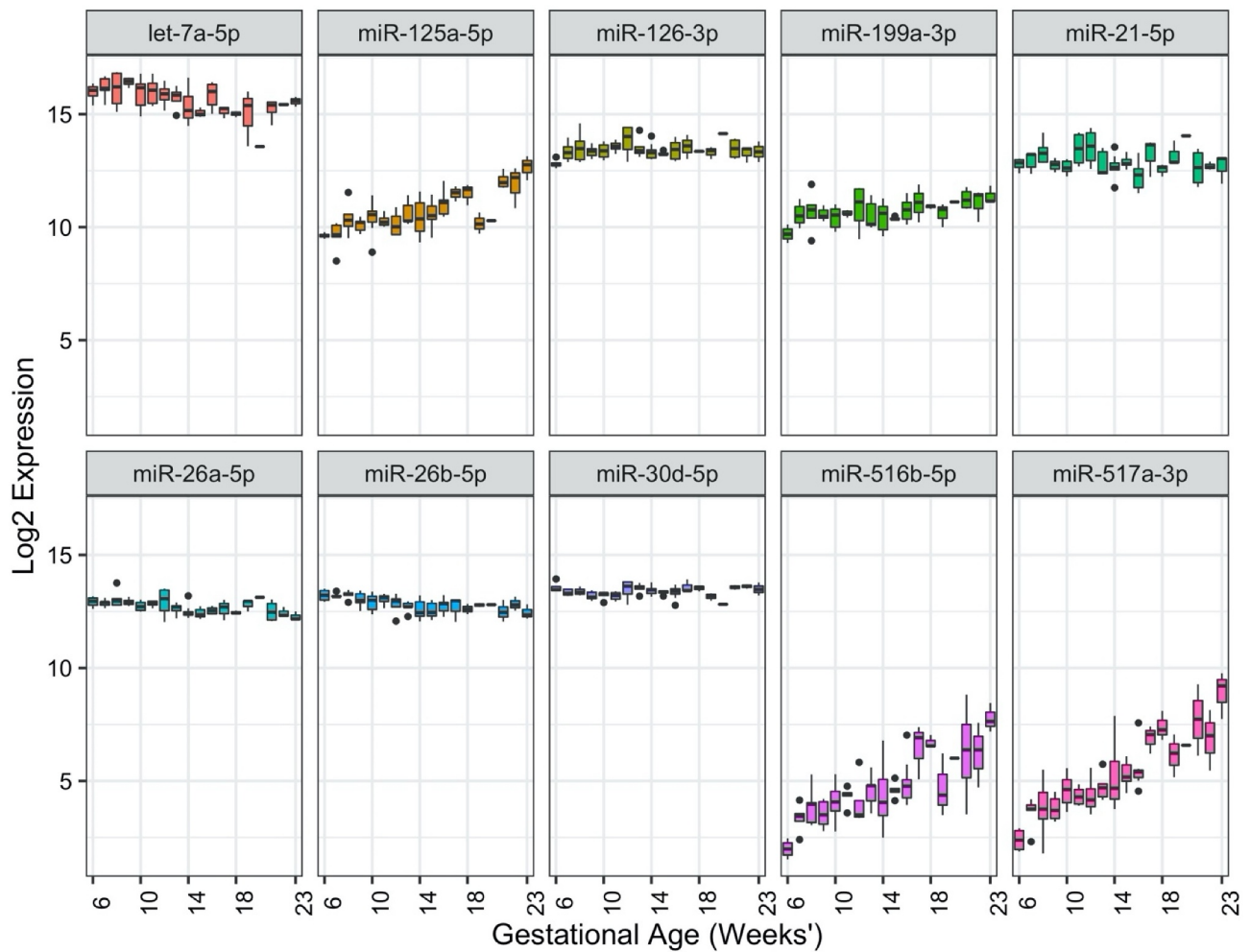
Figure 6. Abundance of maternal plasma miRNAs, identified as highly expressed in chorionic villous tissue, from 6–23 weeks' gestation. All ten miRNAs identified as the most highly expressed in chorionic villous tissue were identified as highly abundant in matched plasma samples. Abundance of the C19MC miRNA members, miR-516b-5p and miR-517a-3p, appear to increase across early to mid gestation.

identified up-regulation of all six miR-34 family members reflecting a reduction in cellular proliferation as the pregnancy progresses and placental growth trajectory begins to slow. Similarly, miR-378a-5p was previously found to enhance cell survival and promote trophoblast migration and invasion [60]. Our data showed significant down-regulation of the miR-34 family in 11–23 weeks' gestation compared to the 6–10 weeks' gestation placenta potentially linking down-regulation of these miRNAs with the reduced trophoblast migration as pregnancy progresses. miR-200 family members are also known to have an established role in placental function, in particular in the cellular transition from epithelial to mesenchymal (EMT) phenotype which is a prerequisite for extravillous trophoblast invasion of the maternal decidua. miR-200 family members play a well-known role in EMT through a feedback loop with the ZEB family of transcription factors. We identified seven members of the miR-200 family, six of which are down-regulated in the 11–23 weeks' gestation group. The miR-200 family is known to be highly expressed in epithelial cell types [61] and thus its down-regulation is consistent with development of the vascularized mesenchymal core in chorionic villi in the second trimester.

Proportional change in expression miRNA clusters play a role in placenta gene regulation during development

miRNA clusters are known to play an important role in the placenta, with these regions thought to have co-evolved and to act cooperatively to repress target genes [62]. Interestingly, while miRNA clusters are typically transcribed as a single polycistronic transcript, we identified large intra-sample variances in the expression levels of cluster members (Additional file 2, Figure S4) hinting at additional post transcriptional regulatory mechanisms. There are a number of miRNA clusters expressed either exclusively, or preferentially in placenta [31,63,64]. In particular, the C19MC, C14MC and miR-17 ~ 92 clusters have been shown to regulate phenotypic and functional diversity in frequently used cell lines and isolated primary trophoblasts, and in the immune system, by which they may play a role in immune tolerance to paternal antigens [32,65,66].

C19MC is expressed almost exclusively in the placenta but also in certain tumours and undifferentiated cells [34,67]. C19MC is a large placenta specific imprinted cluster located on chromosome 19q13.41 and mono-allelically

expressed from the paternally inherited chromosome [68]. This cluster has been difficult to study as its expression is both placenta and primate specific [68] and hence lacks an ortholog in mouse [35]. Importantly, C19MC members are expressed at lower than trophoblast levels in JEG-3 and JAR cells, two cell lines derived from choriocarcinoma [69], and are not expressed in the commonly used HTR8/SVneo cell line [64,67] highlighting the need for primary placental tissue in research. Consistent with our findings, previous research has shown that C19MC constitutes a large proportion of total miRNA transcripts (15% at term), with our data additionally showing a gestational decline from 27% at 6–10 weeks' gestation to 23% at 11–23 weeks' gestation [33,67]. In addition, there was a significant (FDR < 0.05) down-regulation in 34 C19MC cluster members in 11–23 weeks' placenta compared to the 6–10 weeks' gestation. Interestingly, a previous report showed that 46 of the 47 miRNAs from the C19MC cluster were up-regulated in third trimester villous trophoblasts compared to first trimester [32]. It is possible that this miRNA cluster exhibits a dynamic gestational expression pattern but perhaps more likely is the possibility that differences between studies can be explained by differences in expression between assessment of trophoblast versus whole chorionic villous tissue. The latter is more physiologically relevant and fits our question. However, this also introduces the question of whether the changes we see are due at least in part to the change in cell type proportions seen in the placenta throughout gestation [70].

A recent miR-517a/c *in situ* hybridization analysis localized expression from these C19MC miRs to cytotrophoblast and syncytiotrophoblast in chorionic villi, proximal cytotrophoblasts in anchoring villi, decreased expression in distal cytotrophoblasts with further reduction in extra-villous cytotrophoblasts. This finding highlights variable expression of the C19MC cluster in different placental cell types, and shows loss of expression of this cluster as trophoblasts differentiate to extravillous phenotypes post EMT [71]. Another report concurs showing higher expression of C19MC members in villous trophoblasts compared to extravillous trophoblasts suggestive of a role for the C19MC cluster miRNA in attenuation of EVT migration through the direct targeting of mRNA transcripts related to cellular movement [72]. Mouillet *et al.* (2015) suggested a role for C19MC in cellular differentiation or the maintenance of pluripotency [73]. Further investigation into the proportional change in expression from pregnancies complicated by placental pathology may help to identify miRNA species with biomarker potential in plasma. However, low expression levels in some maternal plasma samples have frustrated our efforts in this regard.

C14MC, located at the imprinted DLK1-DIO3 domain on human chromosome 14q32 is the largest known human miRNA cluster comprising 52 miRNA genes expressed from the maternally inherited chromosome [64]. The C14MC cluster is found exclusively in eutherian mammals and has been suggested to be essential to the evolution of this lineage, with expression in humans demonstrating a strong bias towards brain, placenta and some embryonic tissues [63]. Our analysis

identified expression from 84 mature miRNAs in the C14MC cluster with the proportion of member transcripts increased in the 11–23 weeks' gestation group compared to 6–10 weeks' gestation. Previous studies investigating C14MC expression, in both whole villous tissue and in primary cytotrophoblasts, have reported a decrease in expression from first to third trimester. Using whole villous tissue, Gu *et al.* (2013) [74] identified 11 miRNA from this cluster down-regulated in third compared to first trimester, and proposed a link between both C19MC and C14MC in immune suppression and innate/adaptive immune response in the mother. In primary cytotrophoblast, Morales-Prieto *et al.* (2012) [32] identified 34 miRNA from this cluster which were also down-regulated in third compared to first trimester [64,74]. Whilst the discrepancy between our data and Morales-Prieto *et al.* (2013) can be explained by the use of different biological source (single cell type versus whole tissue), that between our data and Gu *et al.* (2013) is not so clear but may be due to differences in technology, the number of cluster members identified, or that our samples range up to 23 weeks' gestation and do not include term. It is plausible that rather than a steady decline from early gestation to term, the expression of C14MC members increases across early gestation, before decreasing at term. Consistent with a recent report by Wommack *et al.* (2018) [75] who profiled circulating miRNAs in maternal plasma and found an inverse relationship between placenta-specific clusters C19MC and C14MC we found this in our tissue samples.

Finally, in the analysis of placenta-associated clusters we investigated the expression of miR-17 ~ 92 and paralogs miR-106a~363 and miR-106b~25, identifying 19 mature miRNAs. The proportion of total expression from miR-17 ~ 92 members and paralogs are less than 1% per cluster, with all three clusters showing an overall decrease in the proportion of total expression across early to mid gestation. Using the miRNA sequences provided by Kumar *et al.* (2013) [65], we determined the 5′ or 3′ origin of the transcripts by cross-checking the given sequences against miRBase v21 [23,65], and were thus able to directly compare their microarray data with our sequencing data (detailed in Additional File 3: Table S11).

Plasma miRNA sequencing offers a potential source of non-invasive biomarkers for detecting placenta health

In addition to establishing the placental miRNA profile across early to mid gestation and, given the potential for placental health biomarker discovery using our access to matched maternal plasma, we also aimed to analyse miRNA profiles in maternal plasma sampled in women at the same time as placenta. The identification of placenta-derived, or pregnancy-associated miRNA in plasma offers a potential window into minimally invasive monitoring of pregnancy progression.

Working with miRNA in plasma is known to be difficult due to potential inclusion of red blood cells or other cellular debris [76–78]. Red blood cell contamination may mask any signal we hoped to detect from the placenta as haemolysis may perturb miRNA species and count distributions due to overloading of red blood cell miRNAs [76,78,79]. In our study we see evidence that 11 of our samples were likely affected by

haemolysis. This common difficulty with plasma data, led us to assess the detection of miRNAs of interest revealing that the 10 most abundant placental miRNAs were reflected by high abundance levels in matched plasma. Furthermore, these plasma miRNAs in our study show strong concordance with previous miRNA plasma studies [77,80].

Differential expression and clear gestational age gradient clustering patterns in placenta miRNAs between 6–10 and 11–23 weeks' gestation were not particularly reflected by maternal plasma, indicating that plasma does not reflect the changes in the miRNA profile in placenta across early to mid gestation. miRNAs that were abundant in placenta were not always found to be abundant in plasma. In particular, miRNAs enriched in Pluripotent stem cell/embryonic stem cell were of low abundance in plasma compared to placenta. With the exception of placenta-specific miRNAs, cellular enrichment could be linked to various other systemic compartments. For example, five miRNAs have cellular enrichment in immune response cells, whilst another three miRNAs have enrichment in pluripotent and embryonic stem cells, and one miRNA is enriched in extra-embryonic stem cells which may originate from the foetal membranes or other extra-embryonic tissue such as chorionic villi. Of particular interest, miR-99a-5p enrichment includes enrichment in mast cells which are involved in inflammatory response and allergic reactions. We see an apparent increase in abundance of miR-99a-5p across early to mid gestation, and some additional increase in male- compared with female-bearing pregnancies, which may be indicative of the increased maternal immune response to the male conceptus [81].

Complementary to the investigation of miRNA clusters in placenta, we also investigated the expression of three highly placenta-associated miRNA clusters and confirmed the abundance of these cluster miRNAs in plasma, with the proportion of cluster member expression revealing biomarker potential in plasma. One pathway for the transfer of placental molecules into the maternal circulation is via exosomes. It has previously been reported that members of the C19MC are the predominant miRNA species expressed in exosomes released from primary human trophoblasts [67]. We were able to confirm the presence of 39 C19MC, 48 C14MC and 20 miR-17 ~ 92 miRNA in matched plasma, with their presence showing promise for the use of miRNA as biomarkers for placental health. Perhaps more importantly, week-by-week examination of miRNAs across early gestation, as described in this study, are key elements for developing pregnancy surveillance measures in the future, especially when combined with disease-specific profiles associated with preeclampsia, gestational diabetes and preterm birth. Our investigation here of miRNAs as biomarkers of placental, and by proxy pregnancy health have identified many areas for further investigation. With the implementation of machine learning algorithms, cell free DNA work and placenta specific analysis standards, much more can be expected from this field.

Conclusion

Appropriate placental growth and development is essential for pregnancy success and miRNAs play roles in mediating these.

We investigated placental and maternal plasma miRNAs using matched samples taken after elective termination of otherwise normal pregnancies and found 374 significantly (FDR <0.05) differentially expressed miRNA between 6–10 and 11–23 weeks' gestation placenta.

The research conducted herein is the most comprehensive attempt to accurately profile the miRNA landscape of the human placenta, in part, because of the use of miRNA sequencing rather than microarray or qPCR. This dataset represents unprecedented access to a large number of human placenta samples from 6–23 weeks of gestation. Together these data are an important reference set for miRNA expression of early to mid gestation placental development and function, at time points rarely seen in previous placental sequencing work. These provide an important resource for the placental biology field. Further investigation using maternal plasma in early gestation samples from pregnancies with known outcomes will provide further insights into the search for miRNAs as biomarkers of pregnancy health and disease.

Authors contributions

CTR created the concept. MS, CTR, JB and TBM conceived and developed experimental plans. TJK, DMcA and DMcC performed experiments. MS and JB analysed the sequencing data with help from KB and QW. Manuscript written by MS, JB and CTR with help from TBM and KP. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The dataset(s) supporting the conclusion of this article are available in NCBI's Gene Expression Omnibus [82] and are accessible through GEO Series accession number GSE151362 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151362).

Declarations

Ethics approval for the collection of placenta tissue and blood from women undergoing elective pregnancy termination between 6–23 weeks' gestation

was provided under HREC/16/TQEH/33, by The Queen Elizabeth Hospital Human Research Ethics Committee (TQEH/LMH/MH).

Disclosure statement

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References

[1] Gude NM, Roberts CT, Kalionis B, et al. Growth and function of the normal human placenta. Thromb Res. 2004;114(5–6):397–407.

[2] Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet. 2005;365(9461):785–799.

[3] Jauniaux E, Watson AL, Hempstock J, et al. Onset of maternal arterial blood flow and placental oxidative stress: a possible factor in human early pregnancy failure. Am J Pathol. 2000;157(6):2111–2122.

[4] Henriksen T, Clausen T. The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. Acta Obstet Gynecol Scand. 2002;81 (2):112–114.

[5] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. [Internet] Cell. 2004;116(2):281–297. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867404000455

[6] Bartel DP. Metazoan MicroRNAs. Cell. 2018;173:20–51.

[7] Winter J, Jung S, Keller S, et al. Many roads to maturity: microRNA biogenesis pathways and their regulation. [Internet] Nat Cell Biol. 2009;11(3):228–234. Available from: http://www.nature.com/articles/ncb0309-228

[8] Ameres SL, Zamore PD. Diversifying microRNA sequence and function. [Internet] Nat Rev Mol Cell Biol. 2013;14(8):475–488. Available from: http://www.nature.com/articles/nrm3611

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

[10] Saini HK, Griffiths-Jones S, Enright AJ. Genomic analysis of human microRNA transcripts. Proc Natl Acad Sci USA. 2007 Nov 6;104(45):17719–24.doi:10.1073/pnas.0703890104

[11] Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. [Internet] Nat Rev Mol Cell Biol. 2009;10(2):126–139.

[12] Dykes IM, Emanueli C. Transcriptional and post-transcriptional gene regulation by long non-coding RNA. [Internet] Genom Proteom Bioinform. 2017;15(3):177–186. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5487525

[13] Morley R. Fetal origins of adult disease. [Internet] Semin Fetal Neonatal Med. 2006;11(2):73–78.

[14] Burton GJ, Fowden AL, Thornburg KL. Placental origins of chronic disease. Physiol Rev. 2016;96(4):1509–1565.

[15] Pantano L, Estivill X, Martí E. SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals ubiquitous miRNA modifications in human embryonic cells. Nucleic Acids Res. [Internet] 2009; 38. Available from: https://watermark.silverchair.com/gkp1127.pdf?token= AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_ 9Cf3qfKAc485ysgAAAbQwggGwBgkqhkiG9w0BBwagggGhMIIB nQIBADCCAZYGCSqGSIb3DQEHATAeBglghkgBZQMEAS4wE QQM1TUUngRW8zyuVolBAgEQgIIBZ7gj8AvZ9tPmZnDNEGC VulK4NZusRxNE3WbfR5NrHrMCKeH

[16] Didion JP, Martin M, Collins FS. Atropos: specific, sensitive, and speedy trimming of sequencing reads. PeerJ. 2017;5:e3720.

[17] Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29:15–21.

[18] Church DM, Schneider VA, Graves T, et al. Modernizing reference genome assemblies. PLoS Biol. 2011;9(7):e1001091.

[19] Andrews S FastQC: a quality control tool for high throughput sequence data. [Internet]. 2010; Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[20] Ewels P, Magnusson M, Lundin S, et al. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32(19):3047–3048.

[21] Pantano L, Estivill X, Martí E. A non-biased framework for the annotation and classification of the non-mirna small RNA transcriptome. Bioinformatics. 2011;27(22):3202–3203.

[22] Griffiths-Jones S, Grocock RJ, van Dongen S, et al. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006;34(90001):D140–4.

[23] Kozomara A, Griffiths-Jones S. MiRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. Internet 2014;42(D1):D68–D73.

[24] Ritchie ME, Phipson B, Wu D, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.

[25] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2009;26(1):139–140.

[26] Law CW, Chen Y, Shi W, et al. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15(2):R29.

[27] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3(1):1–25.

[28] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995;57:289–300.

[29] Wan Q, Yiner-Lee Leemaqz S, Pederson SM, et al. Quality control measures for placental sample purity in DNA methylation array analyses. Placenta. 2019;88:8–11.

[30] Wang W, Feng L, Zhang H, et al. Preeclampsia up-regulates angiogenesis-associated MicroRNA (i.e. miR-17, −20a, and −20b) That target Ephrin-B2 and EPHB4 in human placenta. J Clin Endocrinol Metab. 2012;97(6):E1051–E1059.

[31] Luo -S-S, Ishibashi O, Ishikawa G, et al. Human villous trophoblasts express and secrete placenta-specific MicroRNAs into maternal circulation via exosomes1. Biol Reprod. 2009;81(4):717–729.

[32] Morales-Prieto DM, Chaiwangyen W, Ospina-Prieto S, et al. MicroRNA expression profiles of trophoblastic cells. Placenta. 2012;33(9):725–734.

[33] Carreras-Badosa G, Bonmat A, Ortega FJ, et al. Dysregulation of placental miRNA in maternal obesity is associated with pre-and postnatal growth. J Clin Endocrinol Metab. 2017;102(7):2584–2594.

[34] Bentwich I, Avniel A, Karov Y, et al. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet. 2005;37(7):766–770.

[35] Morales-Prieto DM, Ospina-Prieto S, Schmidt A, et al. Elsevier trophoblast research award lecture: origin, evolution and future of placenta miRNAs. Placenta. [Internet] 2014; 35:S39–S45.

[36] Olive V, Jiang I, He L. Mir-17-92, a cluster of miRNAs in the midst of the cancer network. Int J Biochem Cell Biol. 2010;42(8):1348–1354.

[37] Bortolin-Cavaille ML, Dance M, Weber M, et al. C19MC microRNAs are processed from introns of large Pol-II, non-protein-coding transcripts. Nucleic Acids Res. [Internet] 2009;37(10):3464–3473.

[38] Seitz H, Royo H, Bortolin ML, et al. A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. Genome Res. 2004;14(9):1741–1748.

[39] Schmidt JK, Block LN, Golos TG. Defining the rhesus macaque placental miRNAome: conservation of expression of placental miRNA clusters between the macaque and human. Placenta. 2018;65:55–64.

[40] Chim SSC, Shing TKF, Hung ECW, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem. 2008;54(3):482–490.

[41] Zhao C, Sun X, Li L. Biogenesis and function of extracellular miRNAs. [Internet] ExRNA. 2019;1(1). DOI:10.1186/s41544-019-0039-4.

[42] Mouillet JF, Chu T, Hubel CA, et al. The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction. Placenta. 2010;31(9):781–784.

[43] Mouillet JF, Chu T, Nelson DM, et al. MiR-205 silences MED1 in hypoxic primary human trophoblasts. FASEB J. 2010;24(6):2030–2039.

[44] Shen J, Xia W, Khotskaya YB, et al. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature. 2013;497(7449):383–387.

[45] Camps C, Saini HK, Mole DR, et al. Integrated analysis of microRNA and mRNA expression and association with HIF binding reveals the complexity of microRNA expression regulation under hypoxia. Mol Cancer. 2014;13(1):28.

[46] Mori A, Nishi H, Sasaki T, et al. HLA-G expression is regulated by miR-365 in trophoblasts under hypoxic conditions. Placenta. 2016;45:37–41.

[47] Chelbi ST, Vaiman D. Genetic and epigenetic factors contribute to the onset of preeclampsia. Mol Cell Endocrinol. 2008;282(1–2):120–129.

[48] Cronqvist T, Saljé K, Familari M, et al. Syncytiotrophoblast vesicles show altered micro-RNA and haemoglobin content after Ex-vivo perfusion of placentas with haemoglobin to mimic preeclampsia. PLoS One. 2014;9(2):e90020.

[49] Li Q, Pan Z, Wang X, et al. miR-125b-1-3p inhibits trophoblast cell invasion by targeting sphingosine-1-phosphate receptor 1 in preeclampsia. Biochem Biophys Res Commun. 2014;453(1):57–63.

[50] Poston L, Raijmakers MTM. Trophoblast oxidative stress, antioxidants and pregnancy outcome—A review. Placenta. 2004;25:S72–8.

[51] Burton GJ, Hempstock J, Jauniaux E. Oxygen, early embryonic metabolism and free radical-mediated embryopathies. Reprod Biomed Online. 2003;6(1):84–96.

[52] Hua Z, Lv Q, Ye W, et al. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS One. 2006;1(1):e116.

[53] Hebert C, Norris K, Scheper MA, et al. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. Mol Cancer. 2007;6(1):5.

[54] Kulshreshtha R, Ferracin M, Wojcik SE, et al. A microRNA signature of hypoxia. Mol Cell Biol. 2007;27(5):1859–1867.

[55] Rane S, He M, Sayed D, et al. Downregulation of MiR-199a derepresses hypoxia-inducible factor-1α and sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circ Res. 2009;104(7):879–886.

[56] Gonsalves CS, Kalra VK. Hypoxia-mediated expression of 5-lipoxygenase-activating protein involves HIF-1alpha and NF-kappaB and microRNAs 135a and 199a-5p. J Immunol. 2010;184(7):3878–3888.

[57] Nallamshetty S, Chan SY, Loscalzo J. Hypoxia: a master regulator of microRNA biogenesis and activity. Free Radic Biol Med. 2013;64:20–30.

[58] Forbes K, Farrokhnia F, Aplin JD, et al. Dicer-dependent miRNAs provide an endogenous restraint on cytotrophoblast proliferation. Placenta. [Internet] 2012;33(7):581–585.

[59] Zhang L, Liao Y, Tang L. MicroRNA-34 family: a potential tumor suppressor and therapeutic candidate in cancer. J Exp Clin Cancer Res. 2019;38(1):53.

[60] Luo L, Ye G, Nadeem L, et al. MicroRNA-378a-5p promotes trophoblast cell survival, migration and invasion by targeting Nodal. J Cell Sci. 2012;125:3124–3132.

[61] Humphries B, Yang C. The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. Oncotarget. 2015;6(9):6472–6498.

[62] Wang Y, Luo J, Zhang H, et al. MicroRNAs in the same clusters evolve to coordinately regulate functionally related genes. Mol Biol Evol. 2016;33(9):2232–2247.

[63] Glazov EA, McWilliam S, Barris WC, et al. Origin, evolution, and biological role of miRNA cluster in DLK-DIO3 genomic region in placental mammals. Mol Biol Evol. 2008;25(5):939–948.

[64] Morales-Prieto DM, Ospina-Prieto S, Chaiwangyen W, et al. Pregnancy-associated miRNA-clusters. J Reprod Immunol. 2013;97(1):51–61.

[65] Kumar P, Luo Y, Tudela C, et al. The c-Myc-Regulated MicroRNA-17 92 (miR-17 92) and miR-106a 363 clusters Target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. Mol Cell Biol. 2013;33(9):1782–1796.

[66] Kuo G, Wu CY, Yang HY. MiR-17-92 cluster and immunity. [Internet] J Formosan Med Assoc. 2019; DOI:10.1016/j.jfma.2018.04.013.

[67] Donker RB, Mouillet JF, Chu T, et al. The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod. 2012;18(8):417–424.

[68] Noguer-Dance M, Abu-Amero S, Al-Khtib M, et al. The primate-specific microRNA gene cluster (C19MC) is imprinted in the placenta. Hum Mol Genet. 2010;19(18):3566–3582.

[69] Lee CQE, Gardner L, Turco M, et al. What is trophoblast? A combination of criteria define human first-trimester trophoblast. Stem Cell Reports. 2016;6(2):257–272.

[70] Burton GJ, Fowden AL. The placenta: a multifaceted, transient organ. Philos Trans R Soc Lond B Biol Sci. 2015;370(1663):20140066.

[71] Mong EF, Yang Y, Akat KM, et al. Chromosome 19 microRNA cluster enhances cell reprogramming by inhibiting epithelial-to-mesenchymal transition. Sci Rep. 2020;10(1):3029.

[72] Xie L, Mouillet JF, Chu T, et al. C19MC microRNAs regulate the migration of human trophoblasts. Endocrinology. 2014;155(12):4975–4985.

[73] Mouillet J-FJ-F, Ouyang Y, Coyne CB, et al. MicroRNAs in placental health and disease. Am J Obstet Gynecol. 2015;213(4):S163–72.

[74] Gu Y, Sun J, Groome LJ, et al. Differential miRNA expression profiles between the first and third trimester human placentas. AJP. 2013;304:E836–43.

[75] Wommack JC, Trzeciakowski JP, Miranda RC, et al. Micro RNA clusters in maternal plasma are associated with preterm birth and infant outcomes. PLoS One. 2018;13(6):e0199029.

[76] Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. [Internet] Nat Rev Genet. 2012;13(5):358–369. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4517822

[77] Blondal T, Jensby Nielsen S, Baker A, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. [Internet] Methods. 2013;59(1):164–169. Available from: https://www.sciencedirect.com/science/article/pii/S1046202312002551

[78] Cheng HH, Yi HS, Kim Y, et al. Plasma processing conditions substantially influence circulating microRNA biomarker levels. PLoS One. 2013;8(6):e64795.

[79] Kirschner MB, Kao SC, Edelman JJ, et al. Haemolysis during sample preparation alters microRNA content of plasma. PLoS One. 2011;6(9):e24145.

[80] Brennan GP, Vitsios DM, Casey S, et al. RNA-sequencing analysis of umbilical cord plasma microRNAs from healthy newborns. PLoS One. 2018;13(12):e0207952.

[81] Clifton VL. Review: sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta. 2010;31(Suppl):S33–9.

[82] Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207–210.

[83] Ludwig N, Leidinger P, Becker K, et al. Distribution of miRNA expression across human tissues. Nucleic Acids Res. 2016;44(8):3865–3877.

[84] Lizio M, Harshbarger J, Abugessaisa I, et al. Update of the FANTOM web resource: high resolution transcriptome of diverse cell types in mammals. Nucleic Acids Res. 2017;45(D1):D737–43.