Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations

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We profiled microRNAs (miRNAs) in cell-free serum and plasma samples from human volunteers using deep sequencing of barcoded small RNA cDNA libraries. By introducing calibrator synthetic oligonucleotides during library preparation, we were able to calculate the total as well as specific concentrations of circulating miRNA. Studying trios of samples from newborn babies and their parents we detected placental-specific miRNA in both maternal and newborn circulations and quantitated the relative contribution of placental miRNAs to the circulating pool of miRNAs. Furthermore, sequence variation in the placental miRNA profiles could be traced to the specific placenta of origin. These deep sequencing profiles, which may serve as a model for tumor or disease detection, allow us to define the repertoire of miRNA abundance in the circulation and potential uses as biomarkers.

pregnancy | cancer

The discovery of novel biomarkers holds the promise of early detection and improved prognosis in many neoplastic and infectious diseases (1–4). A particularly promising class of molecular biomarkers are microRNAs (miRNAs) (5–8). miRNAs are short (22 nt), single-stranded, noncoding RNAs that bind messenger RNA (mRNA) in a sequence-specific manner to regulate gene expression (9, 10). miRNAs play an essential role in fundamental biological processes such as differentiation, proliferation, apoptosis and homeostasis (11). Over 500 miRNAs have been identified in humans (12, 13). Single or multiple miRNA precursors are produced in the nucleus through cleavage of longer transcripts by the endoribonuclease III enzyme, Drosha (14, 15). These products then undergo further processing in the cytoplasm by another RNase III endoribonuclease, Dicer.

Alternate processing versions of the predominant miRNA are termed isomiRs and result in mature miRNAs that can vary from the genomic sequence (16). IsomiRs are products of several processes, including modification of the 3′ end by nucleotide additions (typically adenines or uridines), internal editing of nucleotides, and 5′ end deletion/addition resulting from variability in the precise cleavage site by Drosha and Dicer (17). Changes in the relative distribution of isomiRs have been found in different developmental and disease states. For example, nontemplate additions of adenosines to the 3′ ends of miRNAs are highly abundant early in *Drosophila* development (18); some isomiRs preferentially occur in benign gastric tissue, whereas other isomiRs preferentially occur in gastric tumors (19).

miRNAs have tissue-specific expression patterns and hence are appealing biomarker candidates. miRNAs, including tissue-specific miRNAs, are shed into circulation where they have been detected by sensitive RT-PCR–based amplification and microarray techniques (20, 21). In the area of infectious diseases, viral-specific miRNAs or miRNAs common to a class of microbes can be identified in the blood (22, 23). In cancer, miRNA expression is dysregulated, with some miRNAs normally expressed during embryogenesis being reactivated and other miRNAs with tumor-suppressor activity being down-regulated (24, 25). Expression patterns may serve as a fingerprint of the tumor source and marker of disease aggressiveness (26). However, recent theoretical predictions have suggested that a given tumor would have to reach a diameter of 1 cm, ∼9 y of growth for a typical ovarian cancer, to be detectable using current techniques for detecting circulating biomarkers (27).

To evaluate the potential use of circulating miRNAs as biomarkers of disease, we characterized miRNAs present in circulation using a deep-sequencing approach (28). RNA sequencing allows quantification of miRNAs, identification of isomiRs, and detection of novel miRNA sequences (29). We chose human placenta as a model to study tissue-specific miRNAs in the blood because it is present exclusively during a well defined period, namely pregnancy, is relatively large (400–500 g) and well perfused, receiving 20% of the total maternal cardiac output thereby being more likely to contribute to circulating miRNAs (30). Thus, the placenta could serve as a model for studying solid tumor contribution of miRNA to circulation.

Several pregnancy-related diseases have been associated with changes in the miRNA profile in placenta. Therefore, in addition to serving as a model for solid tumors, detection of placental miRNAs can be used as a biomarker for pregnancy-related disorders. Preeclampsia is a common and potentially serious complication of pregnancy that affects 5–8% of all pregnancies and is characterized by the development of hypertension and proteinuria (31). Although different hypotheses have been put forth, the underlying etiology of preeclampsia is unknown (32). Analysis of miRNAs from formalin-fixed placental specimens of preeclampsia patients has demonstrated altered miRNA profiles (33–35). Deep sequencing of miRNAs obtained from placental tissues suggests that, in the setting of preeclampsia, the isomiR profile is altered with increased 3′ end addition of an adenosine (36). Although these studies provided biological insight, their clinical utility is limited by the necessity of obtaining placental tissue after delivery. Attempts to detect plasma miRNAs in maternal plasma have thus far been limited to microarray and quantitative RT-PCR approaches (28, 37). Detecting placental miRNAs by deep sequencing could direct specific noninvasive diagnosis of these diseases of pregnancy.

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To profile circulating miRNA, we developed a nanogram-scale small RNA deep-sequencing method with sample multiplexing. We applied this method on plasma of men and nonpregnant women as well as pregnant women's plasma, the umbilical cord blood of their offspring, and their placentas. This approach allowed comprehensive characterization of the plasma miRNA constituents and determination of the tissue-specific contribution from placenta.

Results

Sample Statistics. A summary of the collected specimens, total RNA, and miRNA content is shown in Table 1. A full description of individual sample characteristics and small RNA annotation data are provided in *SI Appendix*, Table S1. Median total RNA recovery was 18 ng/mL of maternal plasma [interquartile range (IQR) 14–20], 45 ng/mL of fetal plasma (IQR 34–91), 15 ng/mL of paternal plasma (IQR 13–18), and 23 ng/mL in nonpregnant women's sera or plasma (IQR 22–28). Two fetal plasma samples had outlying RNA content; 500 and 1,250 ng/mL, possibly explained by cell lysis and release of RNA from the placenta or collection of umbilical cord blood close to the placenta resulting in capture of plasma containing a higher concentration of RNA.

Read count per sample, excluding reads corresponding to the calibrator oligoribonucleotides, was 2.1 million (IQR 1.2–3.7 million). Median percentage of small RNA reads corresponding to miRNA was higher in placental tissue samples compared with maternal plasma (95% vs. 69%, $P < 0.0001$), paternal plasma (56%, $P = 0.002$), nonpregnant women's plasma or sera (18%, $P = 0.002$) and fetal plasma (87%, $P = 0.043$) samples. Lower relative miRNA content was accompanied by higher rates of nonmapping RNA sequences in nonplacental samples. Plasma samples had low input of total RNA and, therefore, a higher incidence of adapter–adapter byproducts that are classified as unmappable reads. These byproducts were filtered out from further analysis. The higher amount of input total RNA from placenta tissue compared with blood samples (400-fold) likely resulted in fewer adapter-derived byproducts and lower incidence of unmapped reads.

In terms of absolute abundance, median miRNA recovery (Table 1) was 37 fmol/μg of total RNA in placenta (IQR 28–46), 9 fmol/μg of total RNA in maternal plasma (IQR 5–17), 25 fmol/μg in fetal plasma (IQR 15–35), 9 fmol/μg (IQR 7–12) in paternal plasma and 1 fmol/μg (IQR 1–7) in nonpregnant women's plasma or sera. Correspondingly, median miRNA concentrations were 563 fM in maternal plasma, 2.4 pM in fetal plasma, 250 fM in paternal plasma, and 63 fM in nonpregnant women's plasma or sera.

Identification of Plasma miRNA Clusters Specific to Placenta. miRNA precursor clusters represent groups of miRNA processed from a single cistron-like pri-miRNA transcript (12). To identify miRNA precursor clusters specific to pregnancy, we performed unsupervised hierarchical clustering of miRNAs isolated from the plasma of three mothers, their umbilical cord plasma samples, as well as one father and two nonpregnant women (Fig. 1, *SI Appendix*, Fig. S1, and Dataset S1). Relative levels of miRNA cluster mir-498(46) [where "mir-498" denotes the miRNA cistronic cluster and "(46)" the number of cistron members] and, to a lesser extent, mir-127(8) and mir-134(41), were high in placental tissue and in samples from umbilical cord and mothers but were very low in nonpregnant women and fathers (Fig. 1), a finding consistent with what is known about mir-498 representing a cluster of placenta-specific miRNAs in primates (38). Unsupervised sample clustering also revealed a close association within each group of samples. Umbilical cord miRNA profiles were most closely related to the maternal samples. Tissue expression profiling demonstrated that, although mir-127(8) and mir-134(41) were expressed in liver and kidney, mir-498(46) expression was unique to placenta (*SI Appendix*, Fig. S2).

To examine relatedness between placental miRNA profiles and plasma profiles of mothers and fetuses, we compared RNA from plasma collected from mother, father, and umbilical cord trios to RNA from biopsy samples of the corresponding placentas. miRNA abundance was based on normalized read frequency, where the denominator for normalization was the total number of miRNA reads. Distance mapping based on complete miRNA profiles correctly segregated placentas, maternal plasma, paternal plasma, and fetal plasma samples (Fig. 2 and *SI Appendix*, Fig. S3).

Table 1. Study sample characteristics

Samples			Extraction statistics			miRNA			
Group	*n*	Statistic, percentile	RNA content, ng/mL plasma	Total reads	Unique reads	Reads	% of total reads	Plasma concentration, fM	fmol/mcg of RNA
Placenta	8	25th	NA	2,584,217	113,079	2,271,615	94.4	NA	28.1
		50th	NA	3,213,653	127,909	2,866,397	95.3	NA	37.2
		75th	NA	4,320,407	150,472	3,906,444	95.5	NA	45.6
Mothers' plasma	7	25th	14	893,796	173,900	580,484	50.7	219	4.7
		50th	18	1,461,557	242,325	1,144,456	68.7	563	9.3
		75th	20	3,633,998	588,876	1,432,651	75.4	844	17.1
Fetal plasma	10	25th	34	1,418,002	117,158	1,113,805	82.2	1,359	14.6
		50th	45	2,998,968	144,820	1,619,137	87.5	2,406	24.8
		75th	91	7,491,092	377,490	4,081,198	92.2	8,188	35.0
Fathers' plasma	5	25th	13	1,490,566	280,408	981,143	52.5	250	7.3
		50th	15	2,178,003	362,306	1,122,571	56.1	250	8.9
		75th	18	3,074,738	514,689	1,407,053	62.5	500	11.5
Control plasma*	5	25th	23	1,289,020	267,413	128,434	13.1	63	0.6
		50th	23	1,305,137	283,307	149,013	17.7	63	0.9
		75th	28	2,045,575	329,169	194,526	66.2	375	7.4

Tissue source and RNA content of samples. Total RNA concentrations were determined by Qubit fluorometric dye assay (and 260 nm spectroscopy for placental samples), whereas miRNA content was calculated by comparing counts to the known amount of synthetic calibrator oligonucleotides that were sequenced concomitantly. Complete concentration and read count data for individual samples are provided in *SI Appendix*, Table S1.
*Among nonpregnant control women, two samples were plasma and three samples were sera.

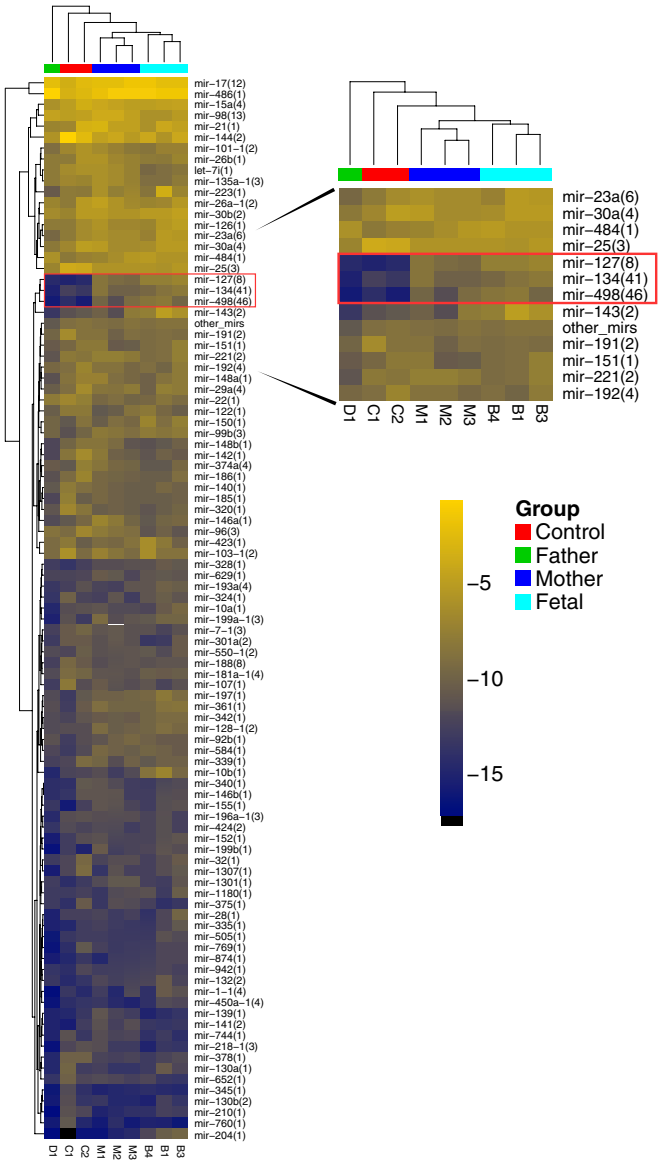

Fig. 1. Mir-498, -127, and -134 clusters are pregnancy-associated. Heatmap of hierarchically clustered plasma samples (columns) and miRNA precursor groups (rows). High relative read frequency (log2-transformed) is indicated by bright yellow shades, low frequencies are shown in dark blue. Corresponding numerical data are presented in Dataset S1.

Similarity was closest between placenta and fetal plasma, followed by placenta and maternal plasma. Paternal and nonpregnant women's plasma samples were most distantly situated. In this trio-based set of samples, 36.7% of all miRNA reads obtained from the placentas were members of the mir-498(46) cluster, and this cluster comprised 0.20% and 1.26% of all reads in maternal and fetal plasma, respectively. In contrast, in paternal plasma, only 0.007% of all miRNA reads were members of the mir-498(46) cluster. Thus, compared with paternal plasma, mir-498(46) members are 5,240-fold more abundant in the placenta, 28-fold more abundant in maternal plasma, and 180-fold more abundant in fetal plasma, in accordance with placental-specific expression of this cluster. This finding implies transmission of placental miRNAs into fetal and maternal circulation.

Individual examination of all mature and star miRNA, and how their corresponding read numbers correlate between placental and plasma samples, revealed uniformly distributed correlation coefficients (Fig. 3*A*), suggesting that among the totality of miRNAs there was no linkage between placentas and corresponding plasma miRNAs. In contrast, limiting the analysis to mir-498(46) cluster members exposed a predominance of positive correlations between maternal plasma or fetal plasma and placental miRNA counts (Fig. 3*B*). Therefore, the abundance of reads annotating to members of the mir-498(46) cluster in plasma samples reflects the specific placenta from which they originate.

Distribution of Placental-Specific miRNA IsomiRs. The miRNA cluster mir-498 codes for 46 distinct miRNAs. Individual miRNAs within the mir-498(46) cluster can be differentially excised and processed at different efficiencies to produce different levels of the constituent miRNAs. Posttranscriptional processing also results in isomiRs of variable length and sequence. We examined the count correlation of the most abundant isomiRs of mir-498 members in placenta (~800 unique sequences) and fetal and maternal plasma samples (Fig. 4*A*). Correlations between placenta and matching plasma were strong, with over 80% of comparisons having *r*-values >0.6 for both mothers and fetuses. This distribution revealed that the isomiR fingerprint within plasma reflected the placenta from which it originated. For perspective, we examined the variant distribution of the miR-98(13) cluster, a collection of multicopy and polycistronic miRNA clusters (miR-125~let-7~miR-99/100). In comparing placentas to fetuses, placentas to mothers, and mothers to fetuses, both the range and median values of the spectrum of correlation coefficients (*r*) were poor in the mir-98 cluster compared with the mir-498 cluster, reflecting the shared origin of the mir-498 cluster member isomiRs (Fig. 4 *B* and *C*). Further investigation of the basis of higher correlation among mir-498 cluster variants revealed that a different degree of 3′ end adenylation of miRNA in placentas, previously suggested to link with pregnancy complications, was captured in respective plasma samples (Fig. 4*D*).

Cluster Member Distribution in Tissue Compared with Plasma. To determine whether miRNA release into plasma, stability in plasma, and clearance from plasma is a sequence-biased or unbiased process, we compared the relative abundance of members of cluster mir-498 in placenta to that of plasma. Fig. 5 shows the read counts for all mir-498 members in the different study groups, normalized to the total number of reads mapping to mir-498(46) (Fig. 5*A*). Differences were seen in the relative levels of mir-498

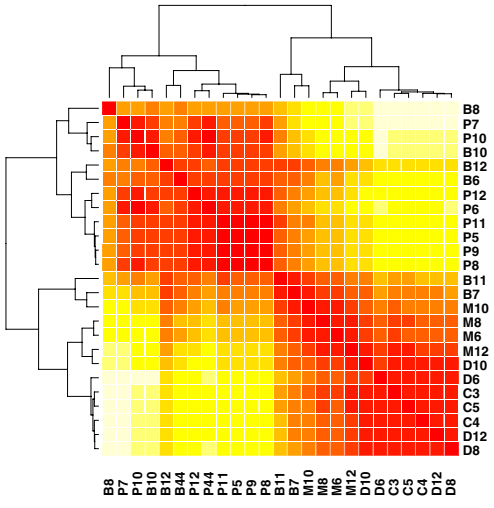

Fig. 2. Placental, fetal, and maternal miRNA profiles are closely related to each other but distantly related to nonpregnant women and paternal samples. Distance mapping of miRNA profiles using Euclidean distance metric of placental biopsy (P) and control plasma (C), paternal plasma (D), maternal plasma (M), and fetal plasma (B) samples is shown. Red color represents a close distance, whereas yellow indicates greater distance. Numeric distance values are shown in *SI Appendix*, Fig. S3.

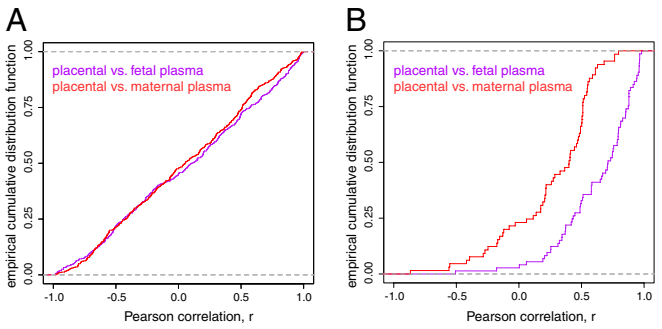

Fig. 3. Mir-498 cluster reads expose association between maternal and fetal plasma miRNA and the specific placenta from which it originated. Pearson correlation of all mature miRNAs (*A*) and mir-498 miRNA(46) members (*B*) between placental and maternal (red) or fetal (purple) plasma samples.

cluster members between tissue and plasma samples, and between plasma samples obtained from fetal blood and maternal blood. Highlighted are several mir-498 cluster members that are preferentially abundant in tissue (Fig. 5*B*) and plasma (Fig. 5*C*). This finding suggests that factors other than biogenesis rates affect the levels of circulating miRNA (e.g., selective secretion and stability), although we cannot exclude the possibility that placental miRNAs in circulation are reflective of the placental tissue closest to the vasculature. Irrespective of the mechanism, these findings should be taken into account when attempting to capture changes in tissue miRNA by studying their circulating counterparts.

Discussion

In this study, we profiled circulating miRNA from blood samples of human volunteers and compared expression levels to that of tissue. An overview inspection of the circulating miRNA profiles revealed that the vast majority of circulating miRNA molecules originate from blood components and endothelial cells. However, tissue-specific miRNAs, for example, from liver and gut, were represented as well, indicating a broad source of tissue contribution to total circulating miRNAs and confirming prior observations (39, 40). During a state of pregnancy, placental miRNAs were also among the more highly detected miRNAs. In contrast, skeletal muscle-, heart-, and most brain-specific miRNA are present at very low levels in healthy plasma, affording a wide dynamic range for potential biomarker applications (*SI Appendix*, Fig. S4).

The 10 most abundant miRNAs in plasma were miR-451-DICER1, miR-486, miR-92a(2), miR-16(2), let-7b, miR-21, miR-19b(2), miR-25, miR-22, and miR-144. miR-451, a Dicer-independent miRNA, is the most abundant miRNA within red blood cells (RBC), comprising ∼50% of the miRNA content in these cells (*SI Appendix*, Fig. S5). Its presence in plasma thus likely represents leakage from RBC or their progenitors, in which it regulates erythroid maturation (41). The second most abundant miRNA in plasma is miR-486, accounting for 13% (IQR 7–30%) of all miRNA (42, 43). It is transcribed from an intron in the ankyrin 1 gene (*ANK1*) expressed in endothelial cells, erythrocyte precursors, and skeletal muscle. It is therefore expected to be detected in plasma, but its plasma levels are higher than other miRNAs that are more abundant in erythrocytes and endothelial cells (e.g., miR-16, miR-144, let-7f), suggesting selective secretion to plasma or increased stability in plasma. Further investigation will be required to elucidate the precise explanation for the vast abundance of this miRNA and the difference in relative abundance seen with this and other miRNA clusters.

The full-term placenta weighs ∼450 g (30). Based on relative read frequencies of our spike-in calibrators, we estimate that the full-term placenta contains 17 nmol of total miRNA and 6 nmol of mir-498(46) members collectively, whereas the circulation of a woman at full term (assuming pregnancy plasma volume of 3.7 L) contains 2.1 pmol total miRNAs and 4.2 fmol of mir-498 (46). Thus, in a woman carrying a full-term pregnancy, there is 1.4×10^6-fold more placental-specific miRNA in her placenta than in her circulation.

We can view the placenta as a model for a solid tumor and members of cluster mir-486 as a model for a tumor-specific miRNA. In our experiments, 1 mg of placenta contains $\sim 2 \times 10^8$ molecules of an individual member of the mir-486 cluster and results in 0.03 molecules of an individual member of mir-486 cluster per mL of plasma. At the technical limits of current day approaches, highly optimized RT-PCR has been able to detect miRNA at a concentration of ∼10 copies per ml. Assuming that a tumor sheds miRNA at similar rate as placenta and that the abundance of the index miRNA is similar to that of a miR-498 member in placenta, a tumor would have to reach a mass of ∼0.3 g to produce sufficient quantities of a tumor-specific miRNA to be theoretically capable of detection by processing 1 mL of plasma and performing RT-PCR. This estimate is similar to the results of mathematical modeling by Hori et al. predicting that an ovarian tumor would have to grow for ∼8.8 y and reach a size of 610 m^3 (0.6 g) to reach the threshold for detection (27). Both our experimental estimates and the mathematical modeling of Hori et al. assume that the biomarker is both unique to the pathology in question and abundant in the source tissue. Techniques allowing capture of more miRNAs from circulation would improve the sensitivity and feasibility of using miRNAs as screening tools. Circulating miRNAs may also prove useful in nononcological setting in which the abundance is higher, such as in Duchenne muscular dystrophy patients (44).

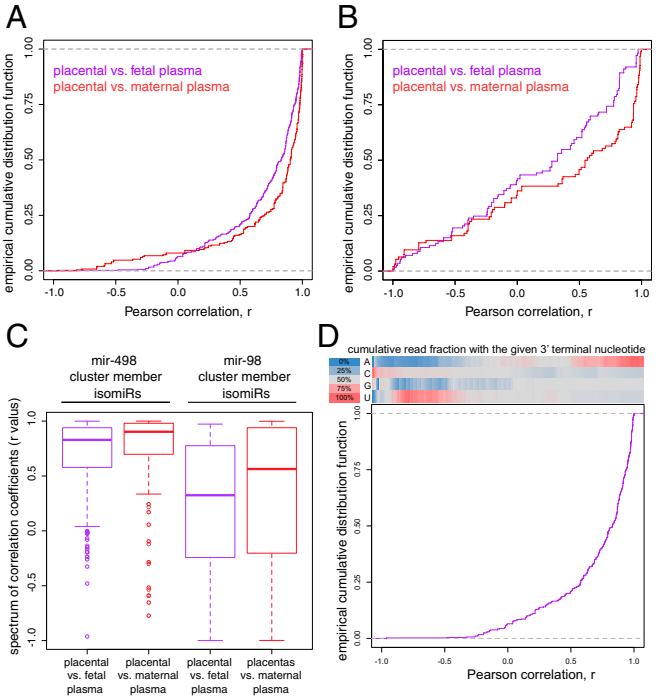

Fig. 4. IsomiRs of mir-498 members, but not mir-98 (miR-125-let-7-miR-99/100) members, uniquely fingerprint the placenta of origin. Pearson correlation of mir-498 member isomiR counts (*A*) and mir-98 member isomiR counts (*B*) between placentas and maternal (red) or fetal (purple) plasma samples. (*C*) Box plots summarizing the distribution of Pearson correlations of miR-498 and miR-98 counts. (*D*) The distribution of Pearson correlations between placental and fetal miR-498 member isomiR reads with reference to 3′ terminal nucleotide (banner) showing that correlation is dependent on the identity of the 3′ terminal nucleotide. The color heatmaps in the banner depict the cumulative read fraction with each 3′ terminal nucleotide along the spectrum of Pearson correlations. Terminal As and Us represented predominantly nontemplated additions. Thus, the highly correlating reads are characterized by a terminal A, whereas the less well correlating reads terminate with G, C, or U.

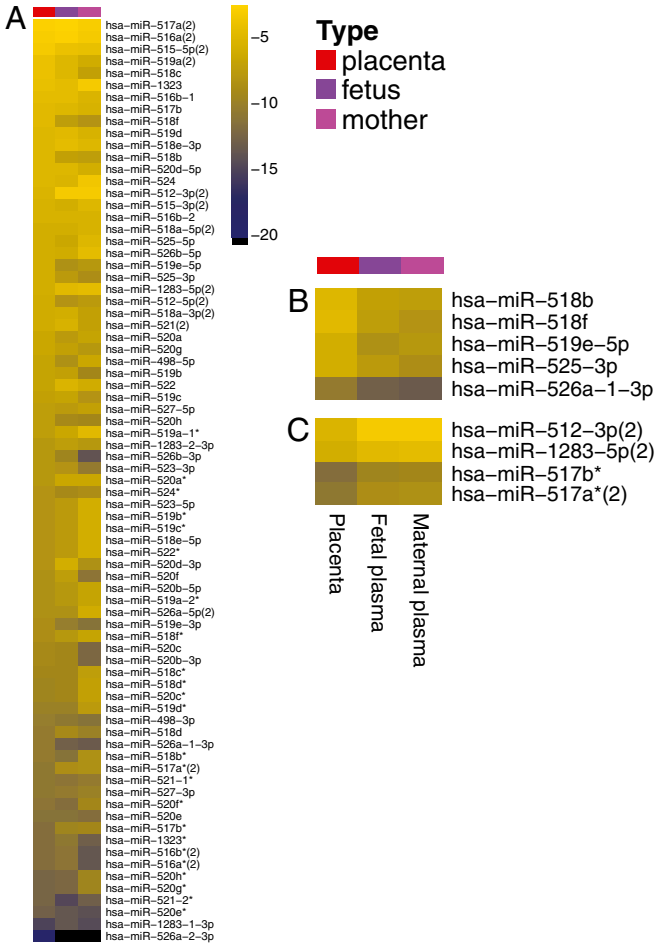

Fig. 5. miRNA cluster members have a different distribution in plasma compared with tissue. (*A*) Heatmap of mir-498 cluster member read counts in pooled study samples. Read counts were pooled from placenta biopsies (red), fetal plasma (purple), and maternal plasma (magenta), and the relative contribution of each cluster member to the total reads in the cluster was calculated. Subsets of miRNA showing relative enrichment (*B*) or depletion (*C*) among cluster members in placenta vs. plasma are depicted separately. Corresponding numerical data are presented in Dataset S1.

Ever since the initial discovery of circulating miRNAs, there has been a question of whether they may exert a hormone-like effect on target tissue (45). Our data argue against this view. The plasma concentration of total miRNA was found to be in the 100 fM range, and any single miRNA was present as a fraction of that concentration. In contrast, steroid hormones such as testosterone, progesterone, and estrogen are present at nM concentrations, whereas even the most trace peptide hormones such as parathyroid hormone and adrenocorticotropic hormone are present in the pM range. Moreover, although typical hormones bind receptors that amplify their signals, miRNAs act on a 1:1 basis with their mRNA targets and are thought to require intracellular levels of greater than ∼1,000 copies per cell to exert measureable activity (38, 46). Thus, in the absence of a known and highly sensitive miRNA receptor, it appears unlikely that miRNAs can function as hormones.

With our methods, placental miRNA fingerprints are sufficiently unique to enable matching of a plasma sample from either the fetus or mother to the specific placenta from which they originated. Moreover, deep sequencing of the plasma miRNAs allows detection of modifications to the miRNA that, in terms of relative frequency, are also specific to the placenta from which they originated. Applying barcoded adapters allows us to multiplex 20 samples in a single sequencing reaction, thereby dramatically lowering the cost (∼75 USD per sample for reagents and sequencing at 5 million reads per sample plus ∼75 USD per sample for labor), limiting batch effects, and improving the prospects for clinical research utility.

Within our pool of samples from healthy volunteers, variability was observed between samples in the relative levels of miRNA and their isomiRs. We were able to use this variability to match the relative miRNA levels and isomiR fingerprints in plasma to the source placenta, suggesting that clinically meaningful changes, such as those observed in preeclampsia, can be detectable in plasma. Therefore, miRNA biomarkers may not only be assessed for the relative abundance of specific miRNAs, but differences in the relative isomiR abundance (or even the presence of a unique isomiR) may also be clinically useful.

Collectively, our results suggest that deep sequencing of barcoded small RNA cDNA libraries from maternal plasma is a robust and relatively inexpensive assay to detect and distinguish tissue- and condition-unique miRNA fingerprints. Further studies will be required using larger sample sizes to determine whether the miRNA profiles from placentas of women with pregnancy complications match the profile seen in maternal plasma and the degree to which a miRNA profile "fingerprint" exists for specific diseases of the placenta. Placental miRNAs in maternal plasma may, in the latter case, be used for noninvasively detecting changes indicative of placental disease in a timely manner so as to allow for closer monitoring or intervention. It will also be of interest to determine whether the altered miRNAs are merely a marker for the placental disease or may participate in its pathogenesis.

Materials and Methods

Patient Samples. EDTA-preserved peripheral blood samples (3 mL) were collected (BD Vacutainer, K2 EDTA, Ref 367856) from pregnant women presenting to the labor floor either for scheduled cesarean section or in labor. Blood samples were concomitantly collected from their respective male partners and, following delivery, from umbilical cord blood (fetal blood). In some cases, a 2-cm section of placenta was collected as well. RNA was isolated from 7 venous blood samples of mothers, 5 venous blood samples from fathers, 10 umbilical cord blood samples ("fetus"), 8 placental biopsy samples, and 5 venous blood samples (3 without anticoagulant, i.e., sera) from nonpregnant women (controls). Blood and placental samples were immediately placed at 4 °C and were processed within 3 h of collection. Blood samples from fathers were selected as controls because processing times and sample conditions could be matched to the maternal and fetal samples. Informed consent was obtained before collecting samples. All aspects of this study were reviewed and approved by the institutional review boards at the Weill-Cornell Medical Center and The Rockefeller University Hospital.

RNA Isolation. Total RNA isolation from placenta biopsy samples was performed with the miRNeasy mini kit (Qiagen) following lysis and homogenization in TRIzol (Invitrogen). Processing of 50 mg of placental tissue with 1 mL of TRIzol yielded ∼56 μg of RNA, as determined by 260-nm spectrometry. For isolation of total RNA from peripheral blood, samples were centrifuged at 500 × *g* (Sorvall Legend RT Rotor 75006441K) for 5 min. Next, 500 μL of plasma (or serum) supernatants were removed, placed in 1.5 mL Eppendorf tubes and centrifuged again at 16,060 × *g* (Sorvall Biofuge fresco) for 5 min to pellet residual cells and debris. A total of 400 μL of supernatant was then transferred to 2-mL Eppendorf tubes. TRIzol LS (1.2 mL) was added and vortexed in each sample. Chloroform (320 μL) was added and mixed. Total RNA was purified using the miRNeasy kit by applying the aqueous phase following 15 min centrifugation at 12,000 × *g* (Sorvall Biofuge fresco) at 4 °C. Total RNA was eluted with 50 μL of water into siliconized (low retention) microcentrifuge tubes (G-Tube Siliconized, Bio Plastic), dried using speed-vacuum, and resuspended in 11 μL of water. One or 2 μL was used to quantify total RNA using Qubit fluorometric dye assay (Invitrogen), which correlated well with quantities reported by picogram-scale capillary electrophoresis (Agilent Bioanalyzer).

cDNA Library Preparation, Sequencing, and Data Analyses. A detailed description of the methods can be found in *SI Appendix* (47). Briefly, a barcoded small RNA cDNA library was prepared from placenta-extracted RNA as described, with 2 μg of total RNA per sample (29). Conversely, 5 ng of total RNA from each plasma or serum sample were used for small RNA cDNA library preparation. When less than 5 ng were obtained, we used the available

amount, taking record for subsequent calculation of quantities. Barcoded 3′-adapters were ligated to RNA in each sample. The 3′-adapter ligation reaction was based on our published method (48), with modifications that adapt to low input RNA quantities: the final concentrations of 3′ adenylated adapters (5 μM) and mutated Rnl2 ligase (0.05 μg/μL) were unchanged; radiolabeled size markers were omitted from the reaction (size identification on subsequent gel purification steps relied on an independently ligated sample of size markers); and a synthetic calibrator mixture consisting of 10 22-nt oligoribonucleotides was added, with sequences noncognate to the human miRNA transcriptome, at a final amount of 0.25 fmol for each of the calibrator oligonucleotides per 1 μg total RNA from the biological sample (0.0125 fmol pooled amount per 3′ ligation reaction on 5 ng total RNA). This mixture allowed absolute quantification of miRNA content in samples within the previously validated molar range (48). Subsequent pooled library preparation steps were performed as described (48). Amplified cDNA libraries underwent 50 cycles of single-end sequencing by synthesis (Illumina GA II or HiSEq. 2000). We used an in-house computer pipeline to extract barcodes, align to the genome (human genome assembly Hg19) and assign small RNA annotations to the reads. miRNA abundance was determined as the sum of all reads with up to two annotation mismatches (49). In-house curated definitions were used for miRNA annotation as well as sequence family and precursor cluster grouping (50). Summary data are expressed as median and IQR unless otherwise stated. Numerical data for data presented in heatmaps is provided in Dataset S1.

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