Clinical Chemistry 54:3
482–490 (2008)

Detection and Characterization of Placental MicroRNAs in Maternal Plasma

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BACKGROUND: The discovery of circulating fetal nucleic acids in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis. MicroRNAs (miRNAs), a class of small RNAs, have been intensely investigated recently because of their important regulatory role in gene expression. Because nucleic acids of placental origin are released into maternal plasma, we hypothesized that miRNAs produced by the placenta would also be released into maternal plasma.

METHODS: We systematically searched for placental miRNAs in maternal plasma to identify miRNAs that were at high concentrations in placentas compared with maternal blood cells and then investigated the stability and filterability of this novel class of pregnancy-associated markers in maternal plasma.

RESULTS: In a panel of TaqMan MicroRNA Assays available for 157 well-established miRNAs, 17 occurred at concentrations >10-fold higher in the placentas than in maternal blood cells and were undetectable in postdelivery maternal plasma. The 4 most abundant of these placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) were detectable in maternal plasma during pregnancy and showed reduced detection rates in postdelivery plasma. The plasma concentration of miR-141 increased as pregnancy progressed into the third trimester. Compared with mRNA encoded by *CSH1* [chorionic somatomammotropin hormone 1 (placental lactogen)], miR-141 was even more stable in maternal plasma, and its concentration did not decrease after filtration.

CONCLUSION: We have demonstrated the existence of placental miRNAs in maternal plasma and provide some information on their stability and physical nature. These findings open up a new class of molecular markers for pregnancy monitoring.

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The discovery of fetal nucleic acids in the plasma of pregnant women *(1–3)* has led to the development of a number of noninvasive prenatal diagnostic tests. Circulating fetal DNA in maternal plasma has been used for prenatal investigations of fetal rhesus D status *(4, 5)*, sex-linked diseases *(6)*, and β-thalassemia *(7)*. Our group has used the epigenetic DNA differences that exist between placenta and maternal blood cells to develop universal fetal-DNA markers *(8–10)* that are sex- and polymorphism-independent. Furthermore, quantitative aberrations of fetus-derived mRNA transcripts have been shown in conditions such as preeclampsia *(11)* and fetal aneuploidies *(12)*. The feasibility of detecting fetal chromosomal aneuploidies in maternal plasma has been demonstrated with a fetus-derived *PLAC4*[4] (placenta-specific 4) mRNA transcript *(13)*. These findings suggest that the detection of circulating fetal nucleic acids holds much promise for noninvasive prenatal diagnosis.

Recent studies on microRNAs (miRNAs)[5] offer possibilities for developing yet another class of molecular markers. miRNAs are short (19–25 nucleotides), single-stranded, and nonprotein-coding RNAs *(14–16)* that regulate gene expression by binding to the 3′ untranslated region of the target mRNAs *(17)* and function in diverse biological processes, including development *(18)*, differentiation *(19)*, apoptosis *(20)*, and oncogenesis *(21, 22)*. Nucleic acids of placental origin were previously shown to be released into maternal plasma *(2, 8)*; hence, it would be interesting to investigate whether miRNAs produced by the placenta are also released into maternal plasma. Because

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Received September 18, 2007; accepted December 21, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.097972
[4] Human genes: *PLAC4*, placenta-specific 4; *CSH1*, chorionic somatomammotropin hormone 1 (placental lactogen); *CGB*, chorionic gonadotropin, beta polypeptide; *CRH*, corticotropin releasing hormone.
[5] Nonstandard abbreviations: miRNA, microRNA; qRT-PCR, quantitative reverse transcription–PCR; IQR, interquartile range.

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ribonuclease activity has been observed in blood *(23)*, however, it has been unclear whether miRNA species in plasma are sufficiently stable to be detected. We describe our systematic search for placental miRNAs in maternal plasma and our investigation into some of the physical properties of the miRNAs we have discovered.

Materials and Methods

PARTICIPANT RECRUITMENT AND SAMPLE COLLECTION

This study was approved by the local institutional review board. Informed consent was obtained from women who had uncomplicated singleton pregnancies and who were patients in the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong. We recruited the first- and second-trimester participants in this study from women attending the antenatal clinic and recruited the third-trimester participants from women undergoing elective cesarean delivery. Samples of maternal peripheral blood (12 mL) were collected into tubes containing EDTA. For the third-trimester participants, we collected placental tissues and postdelivery maternal blood immediately and at 24 h after delivery, respectively.

SAMPLE PROCESSING

To harvest cell-free plasma, we centrifuged maternal blood samples twice at 4 °C. After the first centrifugation at 1600*g* for 10 min, we centrifuged the supernatant at 16 000*g* for 10 min to remove blood cells *(24)*. We harvested maternal blood cells (including leukocytes and erythrocytes) by centrifuging the blood cells obtained in the first centrifugation at 2300*g* for 5 min to remove residual plasma. We then added Trizol LS reagent (Invitrogen) in volumetric ratios of 1:0.8 on 3:1 to the harvested maternal plasma and maternal blood cells, respectively. Placental tissues were preserved in RNAlater (Ambion) immediately following delivery.

RNA EXTRACTION

We extracted total RNA containing small RNA molecules with the Trizol LS or Trizol reagent (Invitrogen) and the *mir*Vana miRNA Isolation Kit (Ambion) (see Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3). After the chloroform-addition steps and phase separation, we mixed the aqueous layer with 1.25 volumes of absolute ethanol, loaded the solution onto the cartridge provided in the *mir*Vana miRNA Isolation Kit, and processed the sample (see Methods in the online Data Supplement). To minimize DNA contamination, we treated the eluted RNA preparation with DNase I (Invitrogen) (see Methods in the online Data Supplement). For miRNA profiling, we further diluted RNA preparations obtained from samples of placentas, maternal blood cells, or postdelivery maternal plasma to 1 mg/L, according to absorbance readings at 260 nm. For the other arms of our study, we did not dilute RNA preparations further.

QUANTIFICATION OF miRNAs BY REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION–PCR ANALYSIS

We used the TaqMan MicroRNA Assay (Applied Biosystems), which has been shown to be highly specific for the intended miRNA but not for its longer preprocessed precursors or for other highly homologous miRNAs that differ in sequence by as little as 1 nucleotide *(25)*. This assay entailed a 2-step quantitative reverse transcription–PCR (qRT-PCR)—reverse transcription of an miRNA of 19–25 nucleotides, priming with a stem-loop primer into a longer cDNA that is amenable to amplification, and quantification by a TaqMan-based qPCR. For each miRNA, we assessed the detection limits of the qRT-PCR assay and quantified the numbers of miRNA copies in samples with a calibration curve (see Methods in the online Data Supplement). miRNA concentrations were expressed as the number of copies per nanogram of RNA extracted from tissue or as the number of copies per liter of plasma. Substrate specificity and assay imprecision also were evaluated for selected miRNAs (see Methods in the online Data Supplement), although the data we obtained cannot be extrapolated directly to all of the other assays.

miRNA PROFILING OF PLACENTAS, MATERNAL BLOOD CELLS, AND POSTDELIVERY MATERNAL PLASMA

We quantified 157 well-established miRNAs in RNA extracts from 5 third-trimester placentas, 5 samples of maternal blood cells, and 5 samples of postdelivery maternal plasma. We used the TaqMan Array Human MicroRNA Panel v1.0 (Early Access) (Applied Biosystems), which contains 157 TaqMan MicroRNA Assays, including the respective reverse-transcription primers, PCR primers, and TaqMan probe. For each assay, we added 2.5 μL (2.5 ng) of the RNA extracted from each of the 15 samples for the reverse-transcription reaction. We used the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) for reverse transcription in a 25 μL of total reaction volume (see Methods in the online Data Supplement). We used the TaqMan Universal PCR Master Mix (Applied Biosystems) for the PCR (see Methods in the online Data Supplement). For each miRNA, we measured the median concentrations in the samples of placenta and maternal blood cells and evaluated the detection rates in the postdelivery samples of maternal plasma (Table 1;

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Table 1. Placental miRNAs identified as candidates for pregnancy-associated markers in maternal plasma.[a]

miRNA	Concentration in placenta, copies/ng extracted RNA	Concentration in maternal blood cells, copies/ng extracted RNA
hsa-miR-141	760 000 (590 000–760 000)	6600 (6000–6800)
hsa-miR-135b	35 000 (29 000–45 000)	0 (0–0)
hsa-miR-149	15 000 (14 000–16 000)	0 (0–0)
hsa-miR-299	11 000 (8300–12 000)	360 (350–710)
hsa-miR-154*	11 000 (8000–11 000)	560 (500–960)
hsa-miR-34c	9100 (6900–11 000)	0 (0–0)
hsa-miR-200b	4100 (2500–5500)	390 (350–500)
hsa-miR-139	3900 (3800–5700)	0 (0–0)
hsa-miR-154	3400 (2900–3400)	210 (170–230)
hsa-miR-368	1900 (1900–2000)	84 (76–170)
hsa-miR-373	1900 (1000–3100)	73 (61–76)
hsa-miR-137	1900 (1600–2400)	0 (0–0)
hsa-miR-184	1800 (1800–2500)	110 (100–160)
hsa-miR-372	960 (240–1700)	0 (0–0)
hsa-miR-371	410 (180–580)	0 (0–0)
hsa-miR-34b	330 (280–460)	30 (0–38)
hsa-miR-337	84 (78–99)	0 (0–0)

[a] Data are presented as the median (IQR). The miRNAs in this table showed both (*a*) concentrations in the placenta >10-fold higher than in maternal blood cells and (*b*) lack of detection (detection rate = 0%) in 5 postdelivery samples of maternal plasma. Other miRNAs not fulfilling both of these criteria are shown in the Table in the online Data Supplement.

see Table in the online Data Supplement). We calculated the fold-change in concentration by dividing the median miRNA concentration in the placenta sample by that in the sample of maternal blood cells.

DETECTION OF PLACENTAL miRNAs IN MATERNAL PLASMA

We conducted TaqMan MicroRNA Assays for miR-16, miR-29a, miR-141, miR-149, miR-299-5p, and miR-135b (Applied Biosystems). To maximize the detection rates of these miRNAs in maternal plasma, we used more concentrated RNA preparations (2.5 μL, no dilution) in the reverse-transcription reaction. The other qRT-PCR steps were the same as those described in the 2 previous sections.

FILTRATION STUDIES OF PLACENTAL miRNA AND mRNA IN MATERNAL PLASMA

To investigate whether the pregnancy-specific miRNA molecules in maternal plasma were associated with subcellular particles, as was previously demonstrated for placental mRNA *(2)*, we filtered samples of maternal plasma. We divided each of 15 processed samples of third-trimester maternal plasma into 4 0.8-mL aliquots. We filtered 3 of the aliquots through a filter with a pore size of 5 μm, 0.45 μm, or 0.22 μm (Millex-GV; Millipore) and left the fourth aliquot unfiltered. We then extracted the RNA from the plasma samples with 1 mL of Trizol LS and quantified the miR-141 concentration in the plasma sample.

STABILITY OF PURIFIED miRNA AND mRNA IN PLASMA

We spiked 210 ng of purified RNA from placental tissues into 6 identical aliquots (0.8 mL each) of a plasma sample freshly collected from a randomly selected male individual and incubated the aliquots at room temperature for 0 s, 5 s, 15 s, 60 s, 1 h, and 2 h. At the end of the incubation period, we immediately added 1 mL Trizol LS reagent to stop any ribonuclease activity and processed the aliquots as described above. We quantified placenta-produced miR-141 and *CSH1* mRNA transcripts in these plasma aliquots according to the methods described above and as previously reported *(2)*. We analyzed another aliquot of this plasma sample with no added placental RNA as a control for the presence of any endogenous *CSH1* transcript and miR-141.

STATISTICAL ANALYSIS

Statistical analyses were performed with SigmaStat 3.0 software (SPSS).

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Results

PRESENCE OF READILY DETECTABLE QUANTITIES OF miR-16 IN MATERNAL PLASMA

miR-16 is ubiquitous in almost all somatic tissues *(26)*, but its presence in plasma has not yet been explored. We detected miR-16 in maternal plasma collected from 6 third-trimester women at a median concentration of 3.4×10^{10} copies/L [interquartile range (IQR), 2.8–3.7×10^{10} copies/L], indicating that miRNA exists in maternal plasma in readily detectable concentrations and validating our protocol for the effective extraction and detection of short RNA species from plasma samples. The SDs for qRT-PCR assays of miR-16 including and excluding the RNA-extraction step were 0.26 and 0.16 threshold cycles (Ct), respectively.

SUBSTRATE SPECIFICITY OF THE TaqMan MicroRNA ASSAY

To rule out the possibility that the TaqMan MicroRNA Assay nonspecifically detects any contaminating genomic DNA in RNA preparations, we treated an RNA preparation with different combinations of DNase I and/or RNase A and assayed for miR-141, which was known to exist at a detectable concentration in this RNA preparation before any treatment. Before any treatment, we detected miR-141 at 2300 copies/ng of extracted RNA. After treatment with DNase I alone, we still detected this miRNA at 2000 copies/ng. After we treated the RNA preparation with RNase A alone or with DNase I plus RNase A, miR-141 decreased to nearly undetectable concentrations (0 copies/ng and 3 copies/ng, respectively). These results suggest that the TaqMan MicroRNA Assay for miR-141 detects RNA but not DNA.

IDENTIFICATION OF PLACENTAL miRNA IN MATERNAL PLASMA

Subsequent to detecting the ubiquitous miR-16 in plasma, we investigated the existence of other of miRNA species in maternal plasma that might be associated with pregnancy. Investigators have previously detected placental mRNA transcripts in maternal plasma, including mRNAs encoded by *CSH1*, *CGB* (chorionic gonadotropin, beta polypeptide), and *CRH* (corticotropin releasing hormone), and have described their rapid clearance from maternal plasma upon delivery of the fetus *(2, 11)*. We hypothesized that placental miRNAs are detectable in maternal plasma and hence profiled the production of 157 miRNAs in 5 third-trimester placentas. By analogy with another previous report that circulating DNA in the plasma of nonpregnant individuals is predominately derived from hematopoietic cells *(27)*, we further hypothesized that the majority of plasma miRNAs that are not associated with pregnancy also originate in the hematopoietic compartment. Because the aim of our study was to identify pregnancy-associated miRNAs in maternal plasma, we compared the miRNA profiles of the placental samples for these 5 pregnancies with the corresponding samples of maternal blood cells. We identified 34 miRNAs that were present in the placenta at concentrations >10-fold higher than in maternal blood cells (Table 1; see Table in the online Data Supplement). Ideally, pregnancy-associated markers should also disappear from the maternal plasma after delivery of the fetus. Hence, we considered only the 17 placental miRNAs that were not detected in the 24-h postdelivery maternal plasma as candidate markers in this phase of the study (Table 1).

DETECTION RATES AND CLEARANCE KINETICS OF PLACENTAL miRNAs IN MATERNAL PLASMA

The detection rates of mRNA transcripts in maternal plasma are reportedly directly related to their concentrations in placental tissues *(28)*. Hence, we reasoned that miRNAs present in high concentrations in the placenta would be more readily detectable in maternal plasma. We therefore investigated whether 4 miRNAs with the highest concentrations in the placenta (miR-141, miR-149, miR-299-5p, and miR-135b) are also present in maternal plasma.

We measured detection rates and clearance kinetics in maternal plasma for the 4 selected miRNAs with a protocol that required a higher initial amount of total RNA. All 4 miRNAs were detected in postpartum maternal plasma at reduced median concentrations and reduced detection rates (Fig. 1, A–D). In particular, the median postpartum concentrations of miR-141 and miR-149 decreased by ≥18-fold. In contrast, we found no systematic change in the concentration of miR-29a, which occurred at similar concentrations in the placenta and maternal blood cells (see Table in the online Data Supplement). miR-29a was used as a positive control for the successful extraction of RNA from all samples (Fig. 1E).

VARIATION OF PLACENTAL miR-141 IN MATERNAL PLASMA WITH GESTATIONAL AGE

Because miR-141 was most readily detected in third-trimester maternal plasma, we investigated its occurrence in plasma during the first and second trimesters. The median gestational ages of the fetuses at the time of blood collection for the first, second, and third trimesters were 13.0 (IQR, 12.8–13.5) weeks, 17.4 (IQR, 17.3–17.6) weeks, and 38.5 (IQR, 38.3–38.6) weeks, respectively. Overall, we observed a trend of increasing miR-141 concentration with gestational age (Fig. 2).

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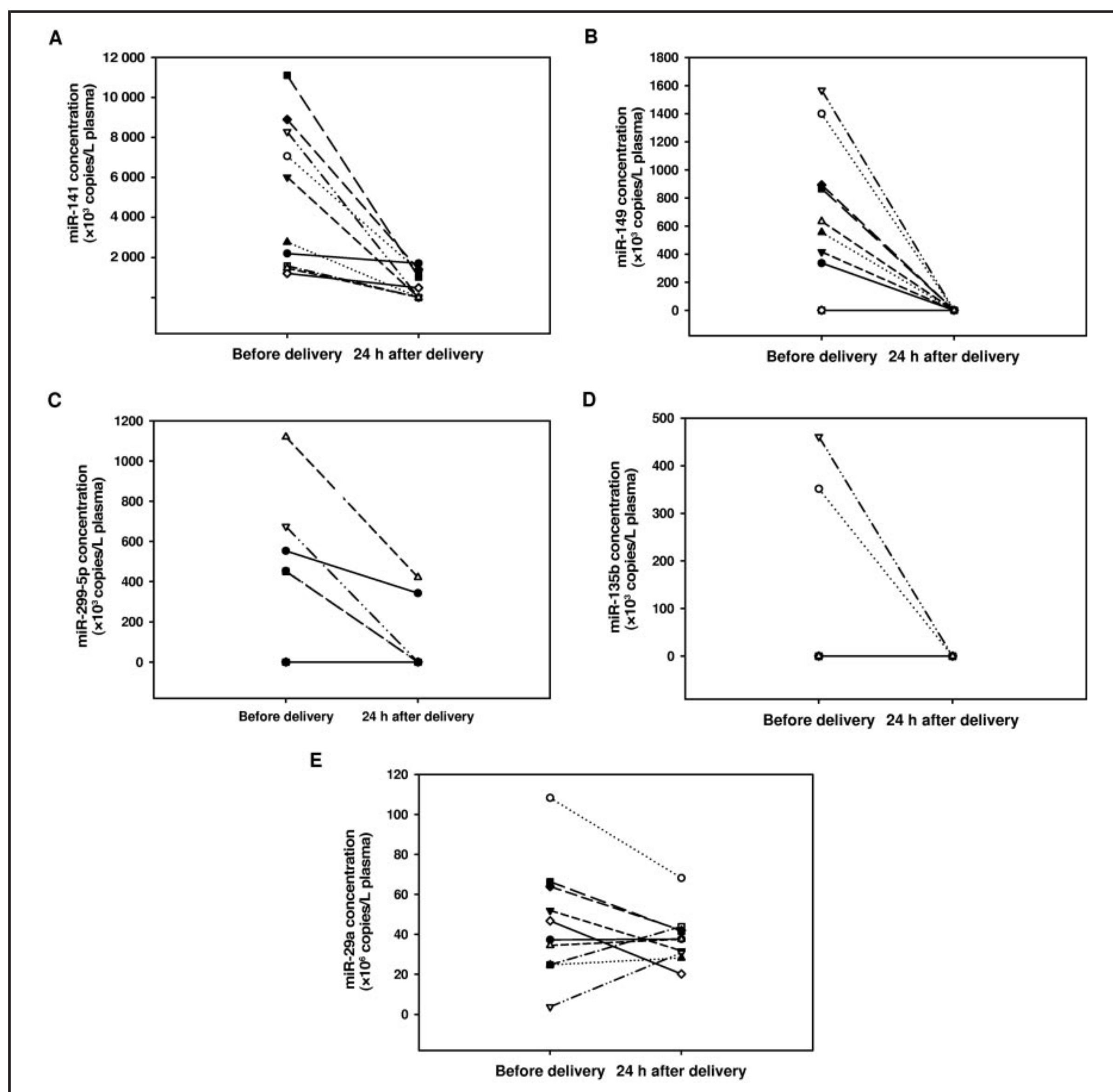

Fig. 1. Concentrations of miR-141 (A), miR-149 (B), miR-299-5p (C), miR-135b (D), and miR-29a (E) in maternal plasma before and at 24 h after delivery.

Pairs of samples from the same pregnancy are depicted by identical symbols connected by a line. A zero value denotes a concentration lower than the respective detection limits: 4×10^5 copies/L for miR-141 and 4×10^4 copies/L for the 4 other miRNAs. The detection rates for the 10 predelivery and 10 postdelivery plasma samples were 100% and 50%, respectively, for miR-141, 80% and 0% for miR-149, 50% and 20% for miR-299-5p, and 20% and 0% for miR-135b. Wilcoxon signed rank tests revealed significant differences in plasma concentrations before and after delivery for miR-141 ($P = 0.002$) and miR-149 ($P = 0.002$), but not for miR-299-5p ($P = 0.063$) and miR-135b ($P = 0.5$). miR-29A was detected in all samples before and after delivery, with no significant change in concentration ($P = 0.432$).

THE EFFECTS OF FILTRATION OF MATERNAL PLASMA ON CIRCULATING PLACENTAL miRNA AND mRNA SPECIES

Because miR-141 was the most readily detectable miRNA in predelivery maternal plasma, we chose this miRNA for filtration studies to further elucidate the molecular characteristics of pregnancy-associated miRNA in maternal plasma. We detected miR-141 in 100% (15 of 15) of the plasma samples in all 4 filtration groups (no filtration or filtration through a 5-μm, 0.45-μm, or 0.22-μm filter) and observed no consis-

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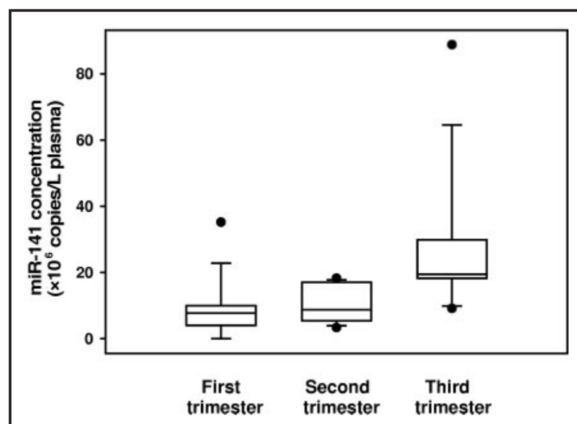

Fig. 2. Concentrations of miR-141 in first-, second-, and third-trimester samples of maternal plasma.

The line within each box denotes the median. The horizontal borders of each box denote the 25th and 75th percentiles, and the limits of the vertical lines ("whiskers") denote the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. A zero value denotes a concentration lower than the miR-141 detection limit of 4×10^5 copies/L. The detection rates were 80%, 100%, and 100% for the 10 plasma samples each obtained at the first, second, and third trimesters, respectively. Significant differences in miR-141 concentrations were noted in first-, second-, and third-trimester plasma samples ($P = 0.002$, Kruskal–Wallis test). Posthoc pairwise comparisons revealed significant differences in plasma miR-141 concentrations between first- and third-trimester samples and between second- and third-trimester samples ($P < 0.05$, Student–Newman–Keuls test). The plasma miR-141 concentration was observed to increase with gestational age (Spearman correlation coefficient, $r, = 0.58$; $P < 0.001$).

tent change in its concentration in any group ($P = 0.257$, Friedman test; Fig. 3A).

In contrast, we detected the *CSH1* transcript, a placental mRNA that reportedly is readily detected in predelivery maternal plasma *(2)*, at reduced rates as the plasma was filtered through filters of increasingly smaller pore size (Fig. 3B). A statistical analysis of the filtration groups showed a significant difference ($P < 0.001$, Friedman test). A pairwise analysis confirmed a statistically significant difference between the no-filter and 0.45-μm filtration groups, and between the no-filter and 0.22-μm filtration groups ($P < 0.05$, Dunn test). Overall, the comparisons of paired samples not filtered or filtered through a 0.45-μm filter showed that the concentration of the *CSH1* transcript decreased by a median of 2.3-fold (IQR, 1.6- to 3.5-fold) in the samples with detectable concentrations.

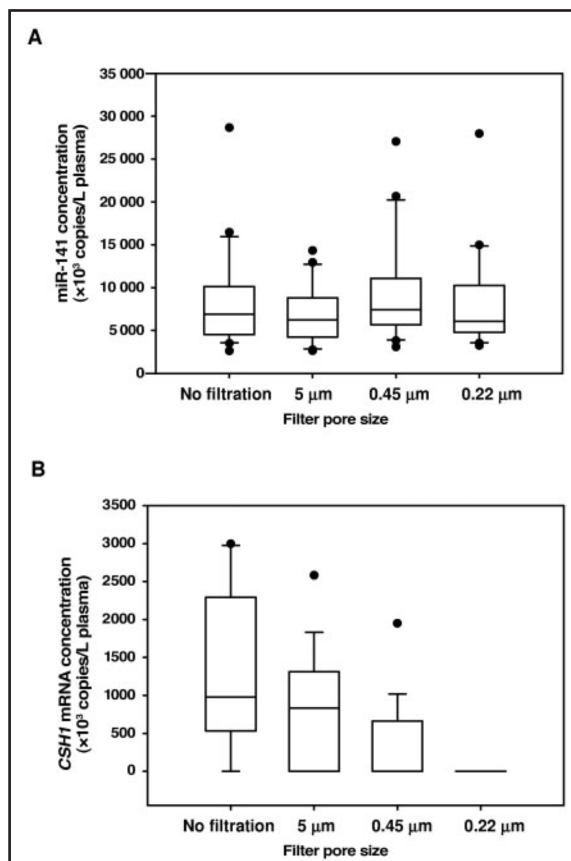

Fig. 3. Concentrations of a placental miRNA (miR-141) (A) and a placental mRNA (*CSH1* transcript) (B) in maternal plasma after no filtration or filtration with filters of different pore sizes.

The horizontal borders of each box denote the 25th and 75th percentiles, and the limits of the vertical lines ("whiskers") denote the 10th and 90th percentiles. Filled circles indicate data points outside the 10th and 90th percentiles. A zero value denotes a concentration lower than the detection limit of 4×10^5 copies/L for miR-141 or 4×10^4 copies/L for the *CSH1* transcript. miR-141 was detected in 100% (15 of 15) of plasma samples in all 4 groups, whereas the *CSH1* transcript was detected in 87%, 60%, 40%, and 0% of the 15 plasma samples in the no-filtration group and the 5-μm, 0.45-μm, and 0.22-μm filter groups, respectively.

STABILITY OF PURIFIED PLACENTAL miRNA AND mRNA IN PLASMA

The different effects of filtration on the concentrations of miR-141 and the *CSH1* transcript in maternal plasma prompted us to further investigate the stability of these 2 different classes of RNA molecules in their purified forms. We purified RNA containing both

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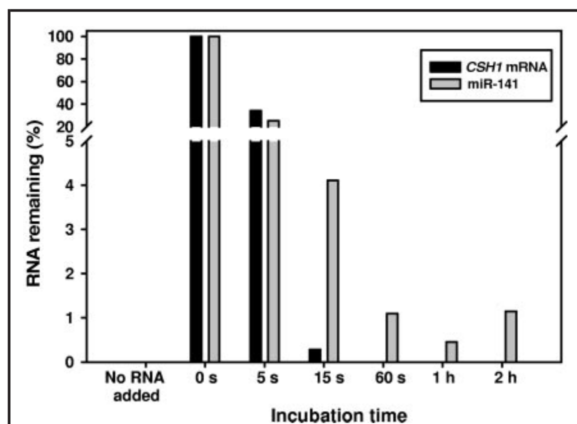

Fig. 4. Percentages of exogenous purified miRNA (miR-141) and *CSH1* mRNA remaining in plasma after incubation for different times.

Concentrations of added miR-141 and *CSH1* mRNA are presented as percentages of the starting concentrations at 0 s of incubation in plasma.

miR-141 and the *CSH1* transcript from the placenta, spiked it into aliquots of plasma from a randomly chosen male individual, and incubated the aliquots for 0 s, 5 s, 15 s, 60 s, 1 h, and 2 h. We did not detect miR-141 and the *CSH1* transcript in the plasma aliquot without the spiked placental RNA (Fig. 4). At the beginning of the incubation period (0 s), the concentrations of miR-141 and the *CSH1* transcript were 1.3×10^9 copies/L and 5.5×10^8 copies/L, respectively; Fig. 4 presents the concentrations of detectable miR-141 and *CSH1* transcript as percentages of these values. In the first 15 s of incubation, the miR-141 concentration decreased 24-fold, from 100.0% to 4.1%. Between 60 s and 2 h of incubation, the miR-141 concentration remained at about 1% (about 10^7 copies/L). In contrast, the concentration of the *CSH1* transcript decreased in the first 15 s by >330-fold, from 100.0% to 0.3%. After 60 s, no *CSH1* transcript could be amplified. We obtained similar results for both miR-141 and the *CSH1* transcript when we repeated this experiment with 110 ng, 430 ng, and 830 ng of placental RNA (data not shown). Thus, the effect was independent of the amount of RNA added.

Discussion

Concordant with our hypothesis that the placenta releases nucleic acids into the maternal plasma, our present data have shown that placental miRNAs exist in maternal plasma in readily detectable quantities. By systematically searching a panel of 157 miRNA assays, we have identified 17 placental miRNAs as candidate markers for monitoring pregnancy in maternal plasma. Furthermore, we detected 4 placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) at higher rates in the maternal plasma before delivery than after delivery. Hence, we conclude that these miRNA species are associated with pregnancy. The highest detection rate and highest median concentration that we observed in predelivery maternal plasma were for miR-141, which was the most abundant placental miRNA detected in this study (Table 1). The reductions in the median concentrations of miR-141 and miR-149 in maternal plasma after delivery were statistically significant, whereas those for miR-299-5p and miR-135b were not. The detection rates for miR-299-5p and miR-135b were ≤50%. This result is consistent with the lower concentrations in the placenta observed for miR-299-5p and miR-135b, compared with miR-141. Thus, these miRNAs were not as readily detectable in maternal plasma as miR-141. We predict that when improved methods for extracting and quantifying miRNA become available, the detection rates for some of these placental miRNAs in maternal plasma will also increase. We also predict that as we expand our search to all of the 530 miRNAs that have been identified in humans to date *(29)*, more pregnancy-associated miRNAs will be identified in maternal plasma.

Our data also have shown that the plasma concentration of a placental miRNA, miR-141, increased as the pregnancy progressed into the third trimester. This increase in miR-141 in maternal plasma may reflect an increase in the size of the placenta or an increased concentration of miR-141 in the third-trimester placenta. The quantification of placental miRNAs in maternal plasma may offer a noninvasive means for monitoring gene regulation in the placenta. Recently, aberrant concentrations of miR-210 and miR-182 were found in preeclamptic placentas delivered at <37 weeks of gestation, compared with the concentrations for non-preeclamptic spontaneous preterm deliveries at matched gestation times *(30)*. It would therefore be useful to investigate whether the aberrant concentrations of miRNAs in placentas involved in preeclampsia and other pathologic conditions are also reflected in maternal plasma.

To develop this novel class of markers for clinical use, we explored the physical nature of a placental miRNA, miR-141, in maternal plasma and compared it with the properties of a placental mRNA, the *CSH1* transcript, which have previously been established *(2)*. The exceptional stability of cell-free mRNA in plasma is probably due to its association with subcellular particles *(2, 31)*, e.g., syncytiotrophoblast microparticles *(32)*. Much to our surprise, however, we were not able to filter out placental miR-141 in maternal plasma, even if 0.22-μm filters were used, in contrast to the

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placental *CSH1* transcript. Hence, unlike the *CSH1* transcript, miR-141 in maternal plasma is not predominantly associated with subcellular particles >0.22 μm in diameter. The question of whether miR-141 is significantly associated with particles <0.22 μm requires further exploration via ultracentrifugation, which can pellet particles the size of viruses.

We further speculated about whether miRNAs themselves are intrinsically more stable in plasma than mRNAs. When we added a purified preparation of exogenous placental RNA to a sample of male plasma with no detectable endogenous miR-141 and *CSH1* transcript, the added miR-141 demonstrated a slower rate of reduction and remained detectable for longer periods than *CSH1* mRNA. Because these data were based on purified miRNA and mRNA in the absence of any protection (e.g., through association with particles) from nuclease activity in the plasma, the higher stability of the former offers an explanation for why miRNA species are readily detectable in plasma even if they are not associated with subcellular particles.

In summary, we have shown that placental miRNAs represent a novel class of fetal nucleic acid markers in maternal plasma. We have also provided the first demonstration of the application of a search strategy for systematically discovering pregnancy-associated miRNAs in maternal plasma. Because miRNAs are exceptionally stable in plasma, they hold promise as markers in the clinical setting. The measurement of miRNAs in maternal plasma for prenatal monitoring and diagnosis would be an interesting future research direction. The biological significance of placental miRNAs in maternal plasma requires further elucidation, but an intriguing possibility is that these small molecules are taken up by cells exposed to the maternal circulation and may modulate gene expression of the maternal compartment. Furthermore, our work has opened up the possibilities that miRNA signatures specific to malignancies *(21, 22)* or viruses *(33)* are also released into the plasma and can be suitable for disease detection and monitoring.

Grant/funding Support: Several of the authors are supported by the Research Grants Council of the Hong Kong Special Administrative Region Government, China (CERG Grant No. CUHK462907, CUHK Direct Grant Nos. 2005.1.027 and 2005.2.010). Y.M.D.L. is supported by the Chair Professorship Scheme of the Li Ka Shing Foundation.

Financial Disclosures: Y.M.D.L., R.W.K.C., and S.S.C.C. hold patents for and have filed patent applications on aspects of the use of fetal nucleic acids in maternal plasma for noninvasive prenatal diagnosis, a proportion of which have been licensed to Sequenom, Inc. Y.M.D.L. is a consultant for Sequenom Inc.

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