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Systematic Search for Placental DNA-Methylation Markers on Chromosome 21: Toward a Maternal Plasma-Based Epigenetic Test for Fetal Trisomy 21

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BACKGROUND: The presence of fetal DNA in maternal plasma represents a source of fetal genetic material for noninvasive prenatal diagnosis; however, the coexisting background maternal DNA complicates the analysis of aneuploidy in such fetal DNA. Recently, the *SERPINB5* gene on chromosome 18 was shown to exhibit different DNA-methylation patterns in the placenta and maternal blood cells, and the allelic ratio for placenta-derived hypomethylated *SERPINB5* in maternal plasma was further shown to be useful for noninvasive detection of fetal trisomy 18.

METHODS: To develop a similar method for the noninvasive detection of trisomy 21, we used methylation-sensitive single nucleotide primer extension and/or bisulfite sequencing to systematically search 114 CpG islands (CGIs)—76% of the 149 CGIs on chromosome 21 identified by bioinformatic criteria—for differentially methylated DNA patterns. The methylation index (MI) of a CpG site was estimated as the proportion of molecules methylated at that site.

RESULTS: We identified 22 CGIs which were shown to contain CpG sites that were either completely unmethylated (MI = 0.00) in maternal blood cells and methylated in the placenta (MI range, 0.22–0.65), or completely methylated (MI = 1.00) in maternal blood cells and hypomethylated in the placenta (MI range, 0.00–0.75). We detected, for the first time, placental DNA-methylation patterns on chromosome 21 in maternal plasma during pregnancy and observed their postpartum clearance.

CONCLUSION: Twenty-two (19%) of the 114 studied CGIs on chromosome 21 showed epigenetic differences between samples of placenta and maternal blood cells; these CGIs may provide a rich source of markers for noninvasive prenatal diagnosis.

Currently, the definitive diagnosis of fetal Down syndrome (trisomy 21), which occurs in 1 of 800 births, requires fetal genetic material to be obtained by invasive procedures that represent a finite risk to the fetus *(1)*. The direct analysis of circulating fetal DNA, which constitutes 3%–6% of the total DNA in maternal plasma *(2, 3)*, for noninvasive diagnosis of chromosome aneuploidies has been complicated by the presence of the coexisting background maternal DNA. Y chromosome sequences or paternally inherited polymorphisms are the most commonly targeted fetal-DNA markers in maternal plasma *(2, 4)*, although no single marker of this kind can be applied to all fetal–maternal pairs (e.g., Y chromosome markers cannot be used for female fetuses).

We have pursued the development of sex- and polymorphism-independent fetal-DNA markers via epigenetic approaches and have used an imprinted locus in selected fetal–maternal pairs to demonstrate the feasibility of this approach for the detection of fetal DNA in maternal plasma *(5)*. To generalize this approach to all pregnancies, we searched for epigenetic differences, namely DNA-methylation differences, between the placenta and maternal blood cells, which have been inferred to be the predominant sources of fetal and maternal nucleic acids, respectively, in maternal plasma *(6, 7)*. Subsequently, we discovered the

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SERPINB5[5] gene [serpin peptidase inhibitor, clade B (ovalbumin), member 5; also known as maspin] to be hypomethylated in the placenta but completely methylated in maternal blood cells. We showed that the placental unmethylated form, U-*SERPINB5*, was released into the maternal plasma during pregnancy and rapidly cleared upon delivery of the fetus. U-*SERPINB5* is thus the first sex- and polymorphism-independent fetal-DNA marker to be found in maternal plasma *(8)*. Because *SERPINB5* is located on chromosome 18, we can infer the dosage of fetal chromosome 18 by assessing the ratio of the polymorphic alleles of fetus-derived U-*SERPINB5* molecules in the maternal plasma. We demonstrated that this epigenetic allelic ratio for U-*SERPINB5* molecules in the maternal plasma of pregnancies involving trisomy 18 was distinguishable from the ratios in unaffected pregnancies *(9)*.

Recently, we have found another fetal epigenetic marker, *RASSF1* [Ras association (RalGDS/AF-6) domain family 1], which was hypermethylated in the placenta but completely unmethylated in maternal blood cells *(10)*. We have developed the placental methylated form, M-*RASSF1*, as a universal marker for fetal DNA in maternal plasma *(11)*. Extrapolating from our results with U-*SERPINB5* and M-*RASSF1*, we reasoned that the placenta and maternal blood cells might also show epigenetic differences on chromosome 21 that may allow us to distinguish fetal and maternal chromosome 21 molecules in maternal plasma; however, DNA-methylation patterns on chromosome 21 have not been investigated systematically for both the placenta and maternal blood cells. One study investigated chromosome 21 methylation in blood cells of nonpregnant individuals *(12)*. Another study investigated chromosome 21 methylation in only 1 male placenta, which served as a control for brain tissues *(13)*. Yet another study investigated chromosome 21 methylation in lung and colon cells *(14)*. A report of a study involving 1 placenta and lymphocytes described the methylation status of chromosomes 6, 20, and 22, but not that of chromosome 21 *(15)*. One last relevant study reported chromosome 21 methylation status in placenta and blood cell samples, but it relied on an assay that used a methylation-sensitive restriction enzyme, *Hpa*II *(16)*, that enables analysis of only 3.9% of all CpG sites in the human genome *(17)*.

Materials and Methods

EXPERIMENTAL DESIGN

We systematically analyzed chromosome 21 methylation in the placenta and maternal blood cells at the resolution of single CpG sites. We analyzed 114 (76%) of the 149 CpG islands (CGIs)[6] identified by bioinformatic means on the long arm of human chromosome 21 *(12)* in the placenta and compared the results with those from maternal blood cells. CGIs containing differentially methylated CpG sites were identified and developed as assays to detect fetal chromosome 21 sequences in maternal plasma.

PARTICIPANT RECRUITMENT, SAMPLE COLLECTION, AND PROCESSING

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. The study was approved by the institutional review board. First- and third-trimester participants were recruited from women undergoing pregnancy termination and elective cesarean delivery, respectively. Maternal peripheral blood samples (12 mL) were collected into tubes containing EDTA just before the obstetric procedures. Placental tissues and postdelivery maternal blood were collected immediately and at 24 h after the procedures, respectively. DNA was extracted from processed samples of placental tissues, maternal blood cells, and plasma (see Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue3).

RAPID METHYLATION ANALYSIS BY METHYLATION-SENSITIVE SINGLE NUCLEOTIDE PRIMER EXTENSION FOLLOWED BY MASS SPECTROMETRIC DETECTION

For the cost-effective and efficient screening of CGIs containing differentially methylated CpG sites in the placenta and maternal blood cells, we used methylation-sensitive single nucleotide primer extension (Ms-SNuPE), a rapid and quantitative method for assessing methylation differences at CpG sites that can be scaled up for high-throughput analysis *(18)*. This method involves a bisulfite treatment that converts unmethylated cytosine residues into uracil residues

[5] Human genes: *SERPINB5*, serpin peptidase inhibitor, clade B (ovalbumin), member 5; *RASSF1*, Ras association (RalGDS/AF-6) domain family 1; *PDE9A*, phosphodiesterase 9A; *C21orf63*, chromosome 21 open reading frame 63; *OLIG2*, oligodendrocyte lineage transcription factor 2; *CBR1*, carbonyl reductase 1; *SIM2*, single-minded homolog 2 (Drosophila); *DSCAM*, Down syndrome cell adhesion molecule; *TRPM2*, transient receptor potential cation channel, subfamily M, member 2; *C21orf29*, chromosome 21 open reading frame 29; *COL18A1*, collagen, type XVIII, alpha 1; *RUNX1*, runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene); *HIST1H2BK*, histone cluster 1, H2bk; *HSF2BP*, heat shock transcription factor 2 binding protein; *COL6A1*, collagen, type VI, alpha 1; *COL6A2*, collagen, type VI, alpha 2.

[6] Nonstandard abbreviations: CGI, CpG island; MI, methylation index; U-*SERPINB5*, unmethylated *SERPINB5*; M-*RASSF1*, methylated *RASSF1*; Ms-SNuPE, methylation-sensitive single nucleotide primer extension; qMSP, real-time quantitative methylation-specific PCR.

and leaves methylated cytosine (5-methylcytosine) residues unchanged *(19)*. Methylated and unmethylated CpG molecules can therefore be distinguished by the changes in base sequence. We targeted such changes by primer-extension reactions to yield products of different masses, which we resolved by matrix-assisted laser-desorption and ionization time-of-flight mass spectrometry with the MassARRAY system (Sequenom; see Methods and Tables S1 and S2 in the online Data Supplement) *(20)*. Unlike methods that use methylation-sensitive restriction enzymes *(12, 13, 16)*, this method can screen for CpG sites that fall outside of any restriction enzyme–recognition sequences. As an illustration, >84% of the CpG sites investigated in our study fell outside the recognition site of *Hpa*II, a commonly used methylation-sensitive restriction enzyme.

We screened 1–3 CpG sites by Ms-SNuPE for each CGI. The methylation index (MI) of each CpG site was estimated by dividing the peak height of the methylated extension product by the total peak height for the methylated and unmethylated products. We performed Ms-SNuPE assays on 5 first-trimester placentas (3 male and 2 female fetuses) and 2 samples of maternal blood cells and reported median MI values for these samples. Ms-SNuPE assays that yielded signals for at least 1 sample from each tissue type were reported for interpretation in this rapid screening.

HIGH-RESOLUTION METHYLATION ANALYSIS BY CLONING AND BISULFITE GENOMIC SEQUENCING

To maximize the chance of identifying CpG sites suitable for marker development, we performed bisulfite sequencing at single-CpG resolution to investigate the methylation status of the dense cluster of CpG sites inside selected CGIs and analyzed >2400 CpG sites. Before sequencing, we performed TA cloning and randomly chose at least 8 clones per PCR to minimize biased representation of any molecules or cell type in each tissue sample (see Methods and Tables S1 and S3 in the online Data Supplement). We calculated the MI for each CpG site by dividing the number of methylated clones by the total number of analyzed clones and expressed MI values for samples of the same tissue type obtained from multiple individuals as the median for each CpG site.

NONINVASIVE DETECTION OF FETAL CHROMOSOME 21 SEQUENCES IN MATERNAL PLASMA

We then explored differentially methylated CpG sites in the placenta and in maternal blood cells as possible fetus-specific DNA markers in maternal plasma. First, we designed real-time quantitative methylation-specific PCR (qMSP) assays *(21)* to target the unmethylated form of CGI084, which is linked to *PDE9A* (phosphodiesterase 9A), in the placenta (U-*PDE9A*) and the methylated form in maternal blood cells (M-*PDE9A*). We designed our second approach, the MassEXTEND assay (Sequenom), to detect the unmethylated form of CGI137 (U-CGI137) in the placenta and assayed for the presence of U-*PDE9A*, M-*PDE9A*, and U-CGI137 in samples of maternal plasma obtained before and after delivery (see Methods in the online Data Supplement).

STATISTICAL ANALYSIS

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

Results

SELECTION OF CHROMOSOME 21 SEQUENCES FOR DNA-METHYLATION ANALYSES

To develop assays for detecting plasma DNA molecules, which are mainly short DNA fragments *(22)*, we looked for loci containing multiple differentially methylated CpG sites within a short stretch of DNA. Thus, CGIs, which contain dense populations of CpG sites, represent valuable targets for our search. For U-*SERPINB5* and M-*RASSF1*, CpG sites suitable for the development of fetal-DNA markers in maternal plasma are either completely methylated or completely unmethylated, respectively, in maternal blood cells *(8, 11)*. This characteristic eliminates the maternal contribution of the targeted DNA species, thus allowing the specific detection of fetal DNA. Of 149 CGIs identified on chromosome 21, 103 and 31 CGIs have previously been reported to be completely unmethylated and completely methylated, respectively, in samples of blood cells obtained from nonpregnant individuals *(12)*. These 134 CGIs were the targets of the current study (see Table S1 in the online Data Supplement). Chromosome 21 sequences containing these 134 CGIs had a median length of 1 834 nucleotides (interquartile range, 1570–2161 nucleotides) and a total of 19 781 CpG sites. We examined these loci for the design of primers for either rapid or high-resolution methylation analyses and determined the placental epigenetic profile for 114 of the 134 targeted CGIs, because most of the remaining 20 CGIs consisted of tandem repeat sequences that proved to be difficult for primer design.

METHYLATION OF ANALYSIS OF CGIs IN MATERNAL BLOOD CELLS BY Ms-SNuPE

We performed an initial study to exclude the possibility that pregnancy might affect the methylation status of blood cells, because a previous study had reported the methylation status of the CGIs on chromosome 21 only in blood cells of nonpregnant individuals *(12)*. We used Ms-SNuPE to study about half of the 114 analyz-

able CGIs in maternal blood cells. Of these 53 CGIs, all of which had previously been reported to be unmethylated *(12)*, we confirmed that 47 (88.7%) of the CGIs contained completely unmethylated (i.e., MI = 0.00) CpG sites in maternal blood cells (Fig. 1A; Fig. S1 in the online Data Supplement). Thus, our data suggest that the methylation status of CGIs in DNA extracted from maternal blood cells is highly concordant with that of CGIs in DNA from blood cells from nonpregnant individuals *(12)*, despite the use of different methods of investigation.

IDENTIFICATION OF CGIs WITH CpG SITES COMPLETELY UNMETHYLATED IN MATERNAL BLOOD CELLS AND PARTIALLY METHYLATED IN THE PLACENTA

Our experience with M-*RASSF1 (10)* indicates that loci that are hypermethylated in the placenta would be promising candidates for fetal-DNA markers if they met 2 criteria: (*a*) criterion 1 [the CpG site is completely unmethylated (MI = 0.00) in maternal blood cells], and (*b*) criterion 2 [the degree of hypermethylation in the placenta reaches an MI of ≥0.20 so as to be detectable in maternal plasma (see Methods in the online Data Supplement)].

We therefore targeted 103 CGIs that had previously been reported to be completely unmethylated in blood cells *(12)* and performed Ms-SNuPE analyses of 5 first-trimester placentas. Our Ms-SNuPE analyses of 53 CGIs revealed that 4 CGIs (7.5%; CGI009, CGI045, CGI071, and CGI113) contained at least 1 CpG site fulfilling both criteria 1 and 2 (Fig. 1A; summarized in Table 1). These data were confirmed by bisulfite sequencing (Fig. 1B and C; Table 2; summarized in Table 1).

For the remaining 50 CGIs not analyzable by Ms-SNuPE, we attempted bisulfite sequencing with samples from 2 first-trimester placentas. Except for CGI138, which failed to yield any products after systematic optimization, we successfully sequenced all 49 of the other CGIs. Eleven (22.4%) of these CGIs contained at least 1 CpG site with an MI ≥0.20 in the placenta, fulfilling criterion 2 (Fig. 1B and C; Table 1), whereas the rest did not (see Fig. 2 in the online Data Supplement). We therefore performed bisulfite sequencing on 2 samples of first-trimester maternal blood cells for these 11 CGIs. Nine (18.4%) of these 49 CGIs contained at least 1 CpG site with an MI of 0.00 in maternal blood cells and an MI ≥0.20 (range, 0.22–0.65) in the placentas, fulfilling both criteria 1 and 2 for a potential fetal marker (Tables 1 and 2).

IDENTIFICATION OF CGIs WITH CpG SITES COMPLETELY METHYLATED IN MATERNAL BLOOD CELLS AND HYPOMETHYLATED IN THE PLACENTA

Our experience with U-*SERPINB5 (8)* suggests that loci hypomethylated in the placenta would be promising candidates for fetal-DNA markers if they fulfilled 2 other criteria: (*i*) criterion 3 [the CpG site is completely methylated (MI = 1.00) in maternal blood cells], and (*ii*) criterion 4 [the CpG site is hypomethylated in the placenta with an MI ≤0.80, to facilitate PCR detection (see Methods in the online Data Supplement)]. Criteria 3 and 4 are essentially the opposite of criteria 1 and 2, respectively.

We therefore targeted the 31 CGIs previously reported to be methylated in blood cells *(12)*; however, we could not analyze 19 of these CGIs because they contained tandem repeat DNA sequences or were highly similar to regions on other chromosomes. Primers for bisulfite sequencing were successfully designed for the remaining 12 CGIs. We analyzed 5 third-trimester samples of placenta and maternal blood cells. Nine (75.0%) of these 12 CGIs contained at least 1 CpG site with an MI ≤0.80 in placental tissues and an MI value of 1.00 in maternal blood cells. (Fig. 1D and E; Tables 1 and 3). Four of these 9 CGIs were chosen for further investigation of 5 paired samples of first-trimester placentas and maternal blood cells, the supply of which was relatively limited compared with third-trimester tissues. We chose CGI084 and CGI137 because they had the lowest median MI values in the third-trimester placentas for the applicable CpG sites. We also chose CGI040 and CGI043 because they had 20 and 21 applicable CpG sites, respectively, that fulfilled criteria 3 and 4 (just a few less than the 25 applicable CpG sites found in CGI084). For the CGI requiring multiple amplicons for analysis, only the amplicon with the most applicable CpG sites was sequenced for the first-trimester tissues. All 4 CGIs also contained CpG sites with an MI value of 1.00 in the first-trimester samples of maternal blood cells and an MI value ≤0.80 in the paired samples of placenta (Fig. 1F and G; Table 3). Thus, these 4 CGIs were suitable for fetal-marker development for both first- and third-trimester maternal plasma.

SELECTION OF CANDIDATE LOCI FOR DEVELOPMENT AS CIRCULATING MARKERS OF FETAL DNA

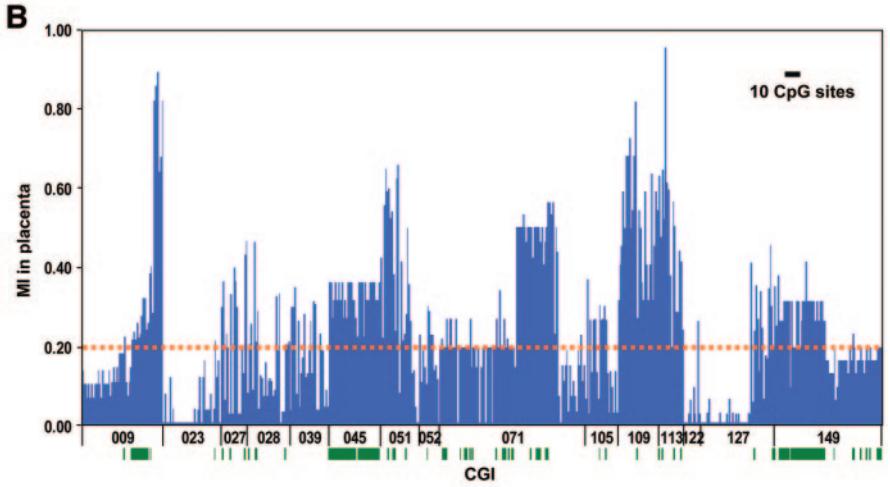
In summary, we identified 22 CGIs (Tables 2 and 3), encompassing 255 CpG sites, which served as suitable candidates for development as markers of circulating fetal DNA. To find the most promising candidates, we considered the CpG sites with the greatest differences in median MI values between the first-trimester placentas and maternal blood cells and selected CGI113 (0.57 vs 0.00), CGI084 (0.50 vs 1.00), and CGI137 (0.50 vs 1.00). To allow the most flexibility in designing assays with short PCR amplicons for detecting short fetal-DNA fragments in maternal plasma *(22)*, we preferred a relatively short distance between consecutive applicable CpG sites. The median (range) distances be-

A

CGI	CpG site no.	MI in maternal blood cells	MI in placenta
009	1*	0.0000	0.7332
	2*	0.0000	0.9214
045	1*	0.0000	0.4043
	2	0.0008	0.4876
	3*	0.0000	0.4758
071	1	0.0282	0.7197
	2	0.1093	0.9101
	3*	0.0000	0.8522
113	1	0.0000	0.1586
	2	0.0768	0.7271
	3*	0.0000	0.6330

Color key for MI values:

0	≤0.2	≤0.4	≤0.6	≤0.8	≤1.0



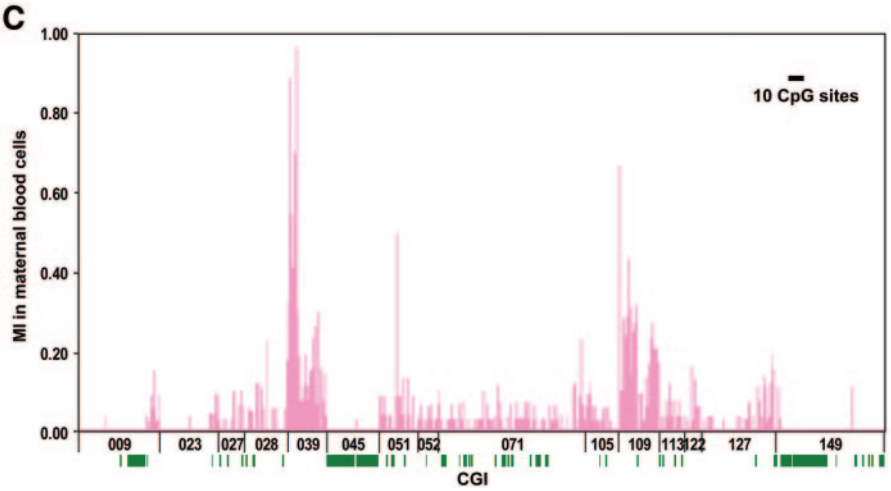


Fig. 1. MIs of independent CpG sites within chromosome 21 CGIs, as measured by Ms-SNuPE (A) and bisulfite sequencing (B–G).

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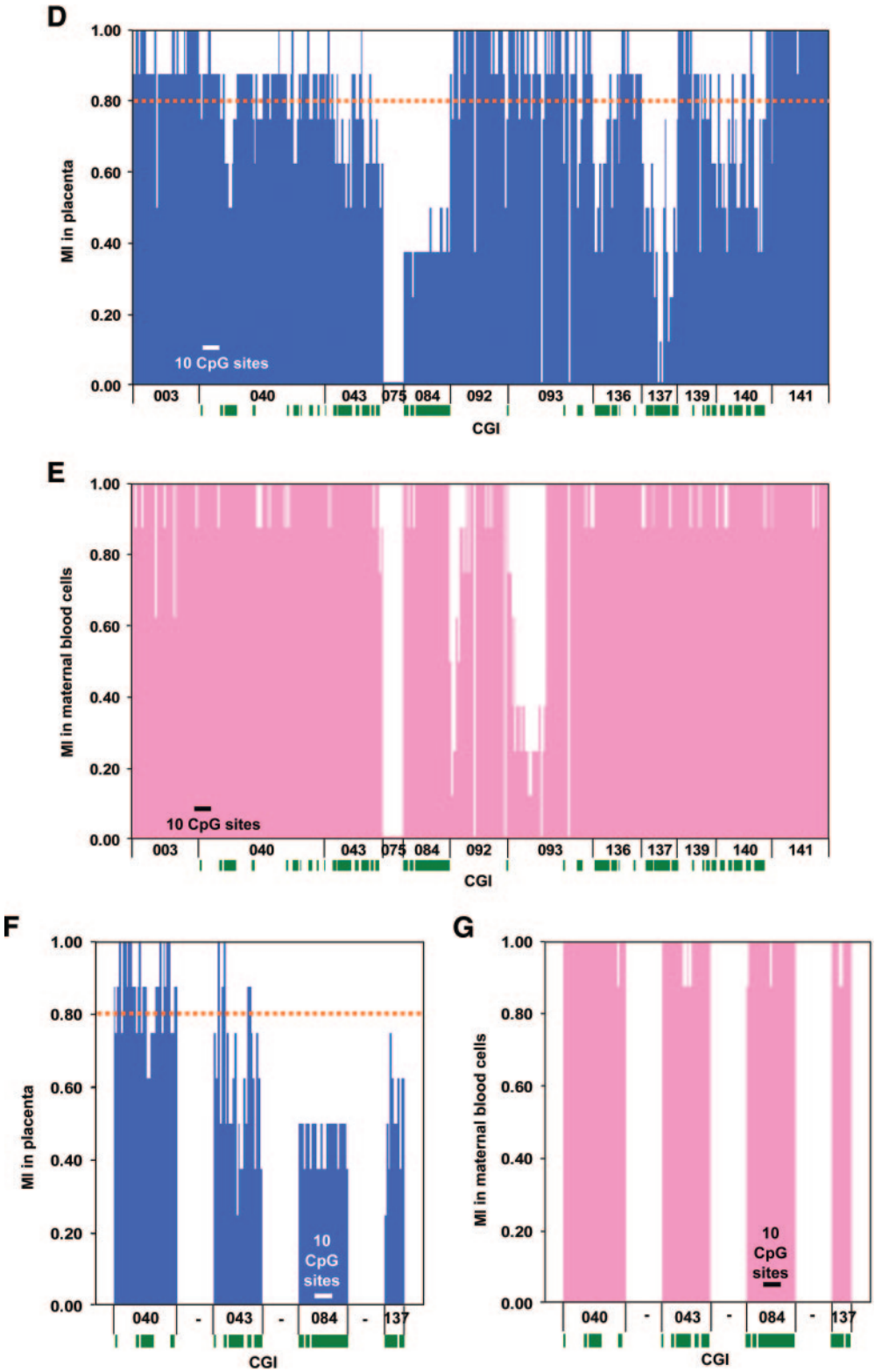

Fig. 1. Continued.

Table 1. Summary of all chromosome 21 CGIs analyzed in this study.

Methylation status in blood cells of nonpregnant individuals[a]	CGIs considered, n	CGIs analyzed, n	CGIs analyzed by methods, n	MI = 0.00 in maternal blood cells, n[b]	MI = 0.00 in maternal blood cells and MI ≥0.20 in placenta, n[b]	MI = 1.00 in maternal blood cells, n[c]	MI = 1.00 in maternal blood cells and MI ≤0.80 in placenta[c]	CGIs applicable for development of fetal-DNA marker, n
Unmethylated	103	102						13
Ms-SNuPE analysis			53	47	4			
Bisulfite sequencing			49	≥9	9			
Methylated	31	12						9
Bisulfite sequencing			12			11	9	
Compositely or incompletely methylated	15	NA[d]						NA
Total	149	114						22

[a] Based on *Hpa*II-McrBC PCR; see Yamada et al. *(12)*.
[b] CGIs with at least 1 CpG site in a CGI fulfilling criterion 1 or both criteria 1 and 2.
[c] CGIs with at least 1 CpG site in a CGI fulfilling criterion 3 or both criteria 3 and 4.
[d] NA, not applicable for fetal-marker development.

tween consecutive applicable CpG sites were 26 (22–101) nucleotides, 8 (2–45) nucleotides, and 13 (3–30) nucleotides in CGI113, CGI084, and CGI137, respectively; hence, we chose CGI084 and CGI137 for further investigation in maternal plasma.

DETECTION OF PLACENTAL U-*PDE9A* DNA BY REAL-TIME qMSP IN MATERNAL PLASMA AND POSTPARTUM CLEARANCE

We hypothesized that DNA sequences bearing placenta-specific epigenetic signatures would be released into maternal plasma and be cleared rapidly after delivery of the fetus. To test this hypothesis, we examined samples of maternal plasma obtained from 12 third-trimester pregnancies (8 male and 4 female fetuses) before and after delivery for the presence of the unmethylated form of CGI084 (U-*PDE9A*), which is linked to the *PDE9A* gene. On the basis of the *PDE9A*-methylation patterns in the placenta and maternal blood cells, we designed a qMSP assay that targeted U-*PDE9A* (Fig. 2A). Before delivery, we detected U-*PDE9A* in the 12 samples of maternal plasma at a median (interquartile range) concentration of 76×10^3 ($38 \times 10^3 - 21 \times$

Fig. 1. Continued. MIs of independent CpG sites within chromosome 21 CGIs, as measured by Ms-SNuPE (A) and bisulfite sequencing (B–G).

CGI is defined according to Yamada et al. *(12)*. (A), Ms-SNuPE revealed 4 CGIs containing CpG sites (asterisks) that fulfill both criteria 1 (MI = 0.00 in maternal blood cells) and 2 (MI ≥0.20 in the placenta) for the development of a fetal marker. CpG sites 1–3 were interrogated by Ms-SNuPE extension primers (see Table S5 in the online Data Supplement). CGIs with no investigated CpG sites fulfilling these criteria are shown in Fig. S1 in the online Data Supplement. *Bar graphs* show MIs measured by bisulfite sequencing for CGIs previously reported to be unmethylated (B, C) and methylated (D–G) in the blood cells of nonpregnant individuals. Each data point represents the MI for 1 CpG site. Tables S2 and S3 in the online Data Supplement detail the genomic location and MI values for each CpG site. MIs in the first-trimester placenta (B) and the first-trimester maternal blood cells (C) are indicated. *Green bars* below the CGI numbers on the *x-axis* indicate CpG sites that fulfill both criteria 1 (MI = 0.00 in maternal blood cells) and 2 (MI ≥0.20 in the placenta, above *orange dotted line* in *panel A*). CGIs lacking any CpG site with an MI ≥0.20 in the placenta are shown in Fig. S2 in the online Data Supplement. MIs in third-trimester placenta (D), third-trimester maternal blood cells (E), first-trimester placenta (F), and first-trimester maternal blood cells (G) are indicated. *Green bars* indicate CpG sites that fulfill both criteria 3 (MI = 1.00 in maternal blood cells) and 4 (MI ≤0.80 in the placenta, below *orange dotted line* in *panels* D and F). By targeting the CpG sites highlighted in the green bars, we can eliminate the maternal DNA sequences in the background and specifically detect fetal DNA sequences in maternal plasma.

Table 2. CGIs suitable for fetal-marker development, with CpG sites completely unmethylated (MI = 0.00) in maternal blood cells.

CGI	CGI-linked gene	CpG sites fulfilling both criteria 1 and 2 in first-trimester tissues, n	MI in first-trimester placenta[a]
009[b]	None	13	0.24 (0.22–0.40)
023	*C21orf63*, chromosome 21 open reading frame 63	1	0.22 (0.22–0.22)
027	None	3	0.37 (0.33–0.43)
028	*OLIG2*, oligodendrocyte lineage transcription factor 2	4	0.21 (0.21–0.29)
045[b]	*CBR1*, carbonyl reductase 1	32	0.36 (0.23–0.36)
051	*SIM2*, single-minded homolog 2 (Drosophila)	4	0.33 (0.24–0.60)
052	*SIM2*	1	0.30 (0.30–0.30)
071[b]	*DSCAM*, Down syndrome cell adhesion molecule	21	0.20 (0.20–0.56)
105	None	2	0.29 (0.27–0.31)
109	*TRPM2*, transient receptor potential cation channel, subfamily M, member 2	1	0.27 (0.27–0.27)
113[b]	*C21orf29*, chromosome 21 open reading frame 29	4	0.57 (0.41–0.65)
127	*COL18A1*, collagen, type XVIII, alpha 1	2	0.22 (0.20–0.24)
149	None	39	0.27 (0.20–0.42)

[a] Data are presented as the median (range).
[b] CGIs initially indicated by Ms-SNuPE to be differentially methylated in maternal blood cells and the placenta.

10^4) copies/L (Fig. 2B). At 24 h postpartum, U-*PDE9A* concentrations in the maternal plasma rapidly declined to almost undetectable levels ($P \leq 0.001$, Wilcoxon signed rank test; Fig. 2C). As a positive control for DNA extraction and bisulfite conversion, M-*PDE9A* (which is not placenta specific) was detected in all 24 maternal plasma samples, with no significant differences between samples ob-

Table 3. CGIs suitable for fetal-marker development, with CpG sites completely methylated (MI = 1.00) in maternal blood cells.

CGI	CGI-linked gene	CpG sites fulfilling both criteria 3 and 4 in third-trimester tissues, n	MI in third-trimester placenta[a]	CpG sites fulfilling both criteria 3 and 4 in first-trimester tissues, n	MI in first-trimester placenta[a]
040	*RUNX1*, runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	20	0.75 (0.50–0.75)	12	0.75 (0.63–0.75)
043	*RUNX1*	21	0.63 (0.50–0.75)	17	0.63 (0.38–0.75)
084	*PDE9A*, phosphodiesterase 9A	25	0.38 (0.25–0.50)	25	0.50 (0.38–0.50)
092	*HIST1H2BK*, histone cluster 1, H2bk	1	0.75 (0.75–0.75)	ND[b]	ND
093	*HSF2BP*, heat shock transcription factor 2 binding protein	4	0.63 (0.50–0.75)	ND	ND
136	*COL6A1*, collagen, type VI, alpha 1	14	0.63 (0.38–0.75)	ND	ND
137	None	16	0.38 (0.00–0.75)	9	0.50 (0.25–0.75)
139	*COL6A2*, collagen, type VI, alpha 2	8	0.75 (0.63–0.75)	ND	ND
140	*COL6A2*	19	0.63 (0.38–0.75)	ND	ND

[a] Data are presented as the median (range).
[b] ND, not determined.

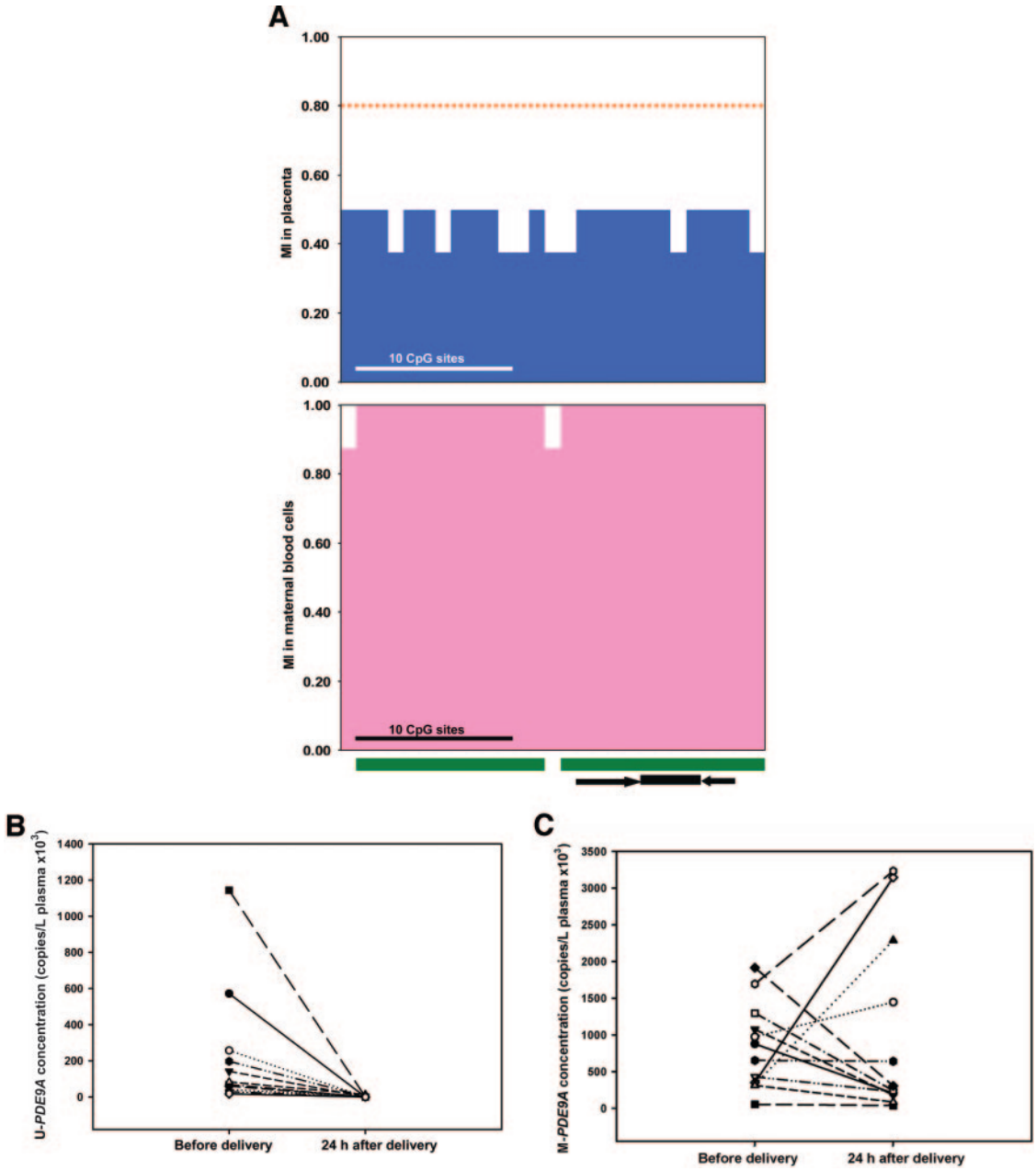

Fig. 2. qMSP assays for U-*PDE9A* and M-*PDE9A*.

(A), the design of the qMSP assay for U-*PDE9A* is based on CpG sites that fulfill both criteria 1 and 2 (*green bars*). *Arrows* show the position of PCR primers. *Black rectangle* shows the position of the TaqMan probe. *Bar graphs* show the MIs measured via bisulfite sequencing for selected CGIs. Each data point represents the MI for 1 CpG site. The genomic location of and MI values for each CpG site are detailed in Tables S2 and S3 in the online Data Supplement. Concentrations of U-*PDE9A* (B) and M-*PDE9A* (C) in the maternal plasma before and after delivery of the fetus are indicated. The lowest detection limits were 6000 copies/L plasma and 16 000 copies/L plasma for the U-*PDE9A* and M-*PDE9A* assays, respectively. A zero value denotes a concentration below these respective limits of detection.

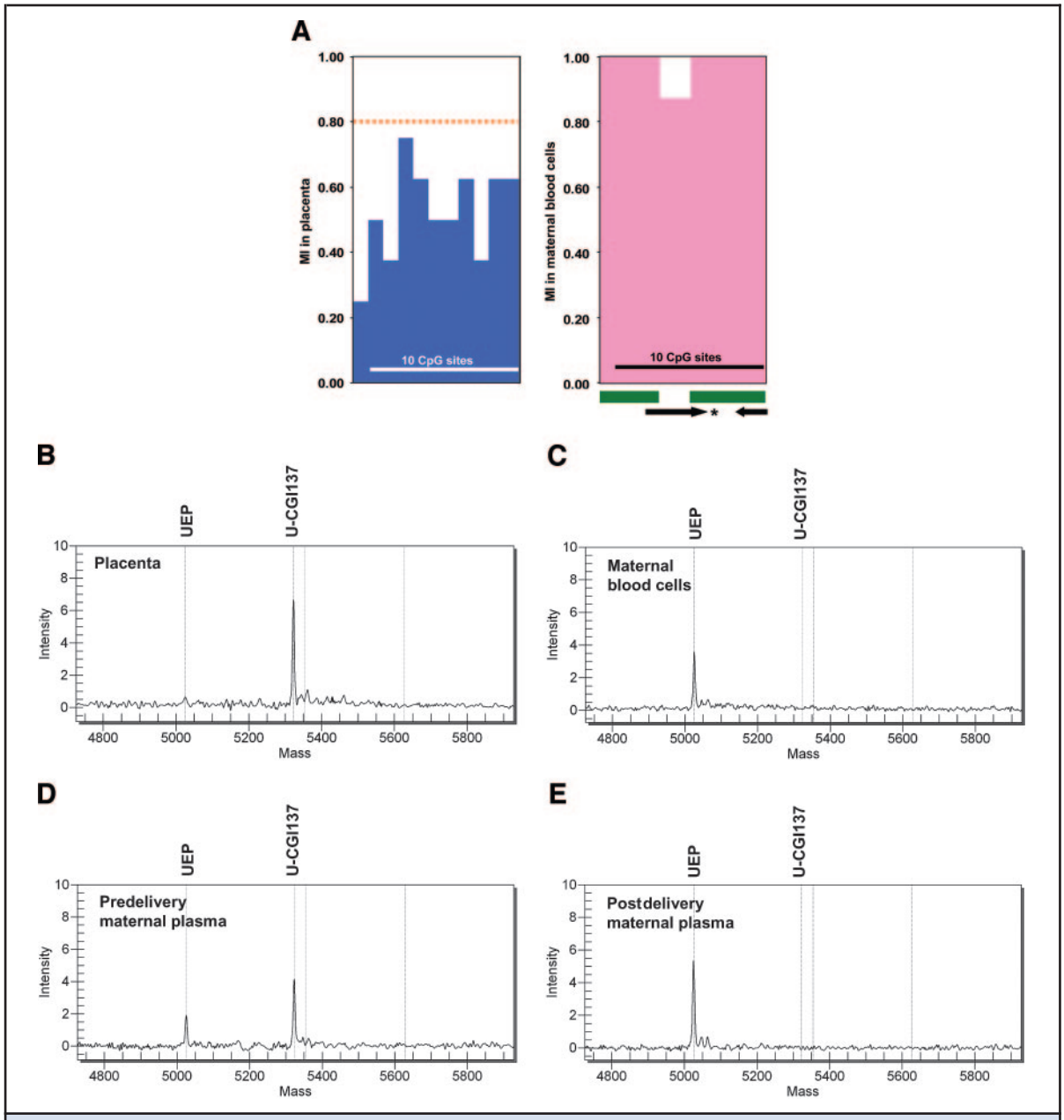

Fig. 3. MassEXTEND assay for detecting U-CGI137.

(A), the design of the MassEXTEND assay for U-CGI137 is based on CpG sites that fulfill both criteria 1 and 2 (*green bars*). *Arrows* show positions of PCR primers. The asterisk shows the CpG site interrogated by the extension primer. *Bar graphs* show the MIs measured by bisulfite sequencing for selected CGIs. Each data point represents the MI of 1 CpG site. Tables S2 and S3 in the online Data Supplement detail the genomic location of and MI values for each CpG site. Indicated are representative mass spectra from the MassEXTEND assay of U-CGI137 in placenta (B), maternal blood cells (C), maternal plasma before delivery of the fetus (D), and maternal plasma after delivery of the fetus (E). For all mass spectra, the *x-axis* depicts the molecular masses of the detected extension products (shown as sharp peaks); the *y-axis* depicts the intensity in arbitrary units. UEP, unextended primer.

tained before and after delivery ($P = 0.910$, Wilcoxon signed rank test).

MassEXTEND DETECTION OF PLACENTAL U-CGI137 DNA IN MATERNAL PLASMA AND ITS POSTPARTUM CLEARANCE

We next investigated the presence of U-CGI137 in maternal plasma; however, we identified far fewer differentially methylated CpG sites in CGI137 than in CGI084 (Table 3). This paucity of differentially methylated sites made it more difficult to develop qMSP assays to distinguish U-CGI137 from M-CGI137. We designed a MassEXTEND assay to detect U-CGI137 after MSP that was based on only 5 identified differentially methylated CpG sites within CGI137, in contrast to 10 such sites in *PDE9A* (cf., Fig. 3A and Fig. 2A), and investigated its specificity and sensitivity for detecting U-CGI137 (see Methods in the online Data Supplement; Fig. 3B and C). In a subsequent application of this assay, we confirmed the presence of U-CGI137 in 8 samples of third-trimester maternal plasma (4 male and 4 female fetuses) obtained before delivery (Fig. 3D). Postdelivery samples of maternal plasma were also available for 7 of these 8 cases, and no U-CGI137 was detected in these 7 samples (Fig. 3E). These data demonstrate that loci with few CpG sites differentially methylated in placenta and maternal blood cells can still be used to distinguish fetal DNA in maternal plasma.

Discussion

We have demonstrated in this study that multiple epigenetic differences exist between a fetal tissue (placenta) and a maternal tissue (blood cells) on chromosome 21. We have discovered that 22 CGIs, containing 255 CpG sites, on chromosome 21 exhibit epigenetic differences that can be exploited for the development of markers for circulating fetal DNA. On the basis of these epigenetic differences, we developed 2 novel fetal-DNA markers, U-*PDE9A* and U-CGI137, that are pregnancy specific in maternal plasma and rapidly clear from the circulation upon delivery of the fetus. These markers are independent of fetal sex and genotype and hence could be applied to all fetal–maternal pairs for noninvasive prenatal detection of fetal sequences on chromosome 21.

Our identification strategy involved the use of a rapid Ms-SNuPE approach and a higher-resolution but more labor-intensive approach via bisulfite sequencing. Because both methods are based on bisulfite conversion, we were able to identify CpG sites that were not recognized by any methylation-sensitive restriction enzyme. In fact, only 40 CpG sites applicable for fetal-marker development (16% of the 255 CpG sites; see Table S4 in the online Data Supplement) would be recognized by *Hpa*II, on which a previous chromosome 21 study relied *(16)*. Thus, our bisulfite-based approach has increased the number of applicable CpG sites by 5-fold or more, compared with an *Hpa*II-based approach.

Of the 2440 CpG sites analyzed in this study, 255 CpG sites were found to be suitable for fetal-marker development. Extrapolating this fraction to the >198 000 CpG sites in the nonrepetitive sequence on chromosome 21 *(17)*, we estimate that there are >20 600 CpG sites suitable for fetal-marker development. More recently developed techniques, such as immunoprecipitation of methylated DNA followed by tiling-array analysis *(14)*, are likely to reveal yet more marker candidates.

We believe that the current study has opened up an important resource for facilitating noninvasive prenatal diagnosis of trisomy 21 with maternal plasma. Recently, the identification of RNA transcripts that are differentially expressed in the placenta has allowed the specific detection of fetal RNA in maternal plasma *(23)*, and the allelic ratio for one such RNA transcript on chromosome 21 has been demonstrated to facilitate the noninvasive detection of trisomy 21 *(24)*. We have demonstrated in the present study that placental epigenetic signatures offer another class of markers besides placental RNA transcripts for distinguishing chromosome 21–derived fetal and maternal genetic material in maternal plasma. We envision that placenta-specific epigenetic signatures on chromosome 21 would supplement placenta-expressed RNA transcripts for facilitating the noninvasive diagnosis of trisomy 21. Although placenta-expressed RNA transcripts are often detected at higher concentrations in the plasma, epigenetic signatures of placental DNA have the desirable feature of being apparently relatively widespread in the human genome [22 (19.3%) of 114 analyzed CGIs on chromosome 21]. Such epigenetic markers can be used for the noninvasive prenatal detection of trisomy 21, either via analysis of epigenetic allelic ratios *(9)* or by direct comparison with a placenta-specific DNA-methylation marker on a reference chromosome. The recent development of digital PCR technologies for noninvasive prenatal diagnosis would allow such analyses to be carried out with high precision *(25)*. Furthermore, the discovery of DNA-methylation abnormalities specific for trisomy 21 in future epigenetic studies would ultimately yield a marker for the disorder that is independent of allelic-ratio or chromosomal-dosage analyses. A combination of markers based on both fetal RNA and fetal DNA may pave the way for the development of a noninvasive test for trisomy 21 that would be applicable to almost all fetal–maternal pairs in the general population.

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