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Short Communication

Enrichment, Immunomorphological, and Genetic Characterization of Fetal Cells Circulating in Maternal Blood

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Fetal cells circulating in the peripheral blood of pregnant women are a potential target for noninvasive genetic analyses. They include epithelial (trophoblastic) cells, which are larger than peripheral blood leukocytes. We enriched circulating trophoblastic cells using the isolation by size of epithelial tumor cells (ISET) method. Peripheral blood was obtained at 11 to 12 weeks of pregnancy. Cells isolated by ISET were stained by hematoxylin and eosin or by immunohistochemistry. Large epithelial cells were microdissected and fetal cell identification was obtained by polymerase chain reaction with short tandem repeats and/or Y-specific primers. By analyzing only 2 ml of blood, we found a variable number (n = 1 to 7) of Y-positive cells (overall 15 of 23) in all of the six mothers carrying a male fetus. In contrast, none of the 26 cells isolated from seven mothers carrying a female fetus scored positive. Eleven cells were analyzed by using short tandem repeat-specific markers: six of them showed a fetal profile and five showed a maternal profile consistently with Y-specific results. Only one-fifth of the single cell DNA was used for fetal cell assessment, leaving enough material for further genetic tests. We also show that the ISET approach allows the performance of fluorescence *in situ* hybridization analyses and the detection of DNA point mutations in single microdissected cells. We conclude that this is a powerful approach to enrich circulating fetal cells and prove their fetal origin, and that it may have implications for noninvasive prenatal diagnosis of genetic disorders. ***(Am J Pathol 2002, 160:51–58)***

The possibility of performing early genetic analyses of fetal cells circulating in the maternal peripheral blood is an important goal for modern obstetrical care.[1,2] In the past, major contributions to this field have been provided by tests performed on whole blood samples or on enriched or purified fetal cell populations.[1] In fact, purity is a critical and key parameter, because it influences the test specificity. Y chromosomal DNA has been detected in maternal samples from 5 to 7 weeks of gestation. The number of circulating fetal cells has also been assessed by Y chromosomal polymerase chain reaction (PCR) on peripheral blood samples without previous cell selection to reduce the risk of losing cells. These studies enabled the observation that the mean number of male cells is around 1 per ml during euploid pregnancies and six times more in women carrying fetuses with Down's syndrome.[3]

Until now, this extremely low number of circulating fetal cells has hampered genetic analysis of individual fetal cells[4–7] and, therefore, the development of noninvasive prenatal testing in clinical practice. Fetal cells include lymphoid and erythroid cells, myeloid precursors, and epithelial (trophoblastic) cells. Epithelial cells are known to be larger in size than peripheral blood leukocytes (PBLs).[8] To enrich fetal cells and target them for genetic tests, we used isolation by size of epithelial tumor cells (ISET),[8] a recently described approach that allows efficient isolation of large cells from the peripheral blood of pregnant women. Single large cells were microdissected

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and their fetal origin assessed using PCR with short tandem repeat (STR)-specific and Y-specific primers.

In this report, we show that the enrichment of fetal cells obtained by using this new approach is higher than that obtained by any other assay reported before. Furthermore, the combination of size-mediated cell enrichment, immunohistochemical characterization, microdissection, and single-cell PCR enables the molecular identification of fetal cells and their genetic characterization.

Materials and Methods

Mothers

We studied 13 women, at 11 to 12 weeks of gestation, carrying fetuses at risk for a genetic disease (six male and seven female fetuses) and recruited after informed consent. Gender diagnosis (DNA analysis) was achieved by chorionic villus sampling.

Methods

Five ml of peripheral blood were obtained on ethylenediaminetetraacetic acid buffer before any invasive procedure and filtered by ISET[8] up to 4 hours after collection. Briefly, blood samples were diluted 1:10 with the filtration buffer and filtered through polycarbonate filters with calibrated, 8-μm-diameter, cylindrical pores. Large cells from each ml of blood were concentrated on a 0.6-cm-diameter circular spot on the filter. Only two spots for each blood sample were analyzed. After hematoxylin and eosin staining or immunohistochemical analysis, the spots were analyzed under the microscope and a picture of each large cell was taken at low and high magnification. Cell size was assessed using Adobe Photoshop software, taking as the reference the 8-μm-diameter of the pores. Pictures were used to recover cells under the PixCell II Arcturus microscope (Mountain View, CA). Laser capture microdissection was performed without any previous treatment of the filter. To ensure that only one cell had been collected, we took pictures of the filter before and after microdissection, and of the microdissected cell on the cap (CapSure HS). The cell was lysed in 15 μl of lysis buffer (100 mmol/L Tris-HCl, pH 8, 400 μg/ml proteinase K) for 16 hours at 37°C. The lysate was collected by centrifugation in a microfuge tube and proteinase K was inactivated at 90°C for 10 minutes. After primer extension preamplification, performed as previously described,[8] the DNA was ethanol precipitated and resuspended in 10 μl of water. Samples were analyzed with HLA primers[8] to check for DNA amplifiability, with STR-specific primers (STR markers D16S3018, forward primer: 5′-6-FAM GGATAAACATAGAGCGACAGzhyTTC-3′; reverse primer: 5′-AGACAGAGTCCCAGGCATT-3′; D16S3031, forward primer: 5′-TET ACTTACCACTGTGCCAGTTG-3′; reverse primer: 5′-ATACATGGGTCCTTAAACCG-3′; D16S539, forward primer: 5′-HEX GATCCCAAGCTCTTCCTCTT-3′; reverse primer: 5′-ACGTTTGTGTGTGCATCTGT-3′) and/or with Y-specific primers (Y1.7 and Y1.8[9]). PCR assays were performed in 20 μl containing 2 μl of the primer extension preamplification product, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.01% gelatin, 200 mmol/L of each deoxynucleotide, 20 pmol of each Y or HLA primer and 1 U of *Taq* polymerase (Perkin-Elmer Cetus, Emeryville, CA). For HLA and Y-specific primers, after the initial denaturation step at 94°C for 5 minutes, 40 cycles were performed (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds), with the final extension step at 72°C for 5 minutes in a Perkin Elmer 9700 thermal cycler. Aliquots (10 μl) of the amplification products were analyzed by electrophoresis on 2% agarose gel. The PCR conditions for Y-specific primers were established by amplifying 5 ng of PBL-derived DNA from three male patients and then by amplifying the primer extension preamplification products obtained from filtered and microdissected HuH6 (hepatocellular carcinoma-derived) cells. For STR-specific PCR, after DNA denaturation at 94°C for 5 minutes, 40 cycles were performed (94°C for 30 seconds, 54°C for 30 seconds, 72°C for 20 seconds) followed by extension at 72°C for 5 minutes. Two μl of the first PCR product were reamplified using the same PCR conditions and profile. One μl of the final PCR product was then mixed with 20 μl of deionized formamide (Sigma-Aldrich, St. Louis, MO) and 0.4 μl of Genescan-500 TAMRA marker and loaded into a ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA). Profiles were analyzed using the Genescan software program (Perkin Elmer, Foster City, CA). Allelotyping was performed by amplifying (using the same STR primers) 1.5 ng of PBL-derived paternal DNA and/or 1.5 ng of PBL-derived maternal DNA obtained before pregnancy and 1.5 ng of trophoblastic DNA obtained by chorionic villus sampling.

Controls of Specificity

The specificity of Y primers was tested by amplifying 10 ng of PBL-derived DNA obtained from 20 women. The precautions taken to avoid carryover of PCR products have been described elsewhere.[10] In addition, a negative control (buffer without sampling) was inserted for each sample at the lysis step and run to the end of the test. When performing laser capture microdissection, we always included at least one microdissection from a new filter (without cells) that was run in parallel with samples and controls. Reamplification with the same Y-specific or STR-specific primers of positive, negative, and control samples was performed to check for the specificity of positive results.

Detection of DNA Mutations in Single Microdissected Cells

We used, as a model, HuH6 cells carrying a point mutation (codon 34: G to T, Gly to Val) in the β-catenin gene. Exon 3 of β-catenin was amplified by a nested protocol using 10 μl of the primer extension preamplification product and 10 pmol of each β-catenin-specific primer (for-

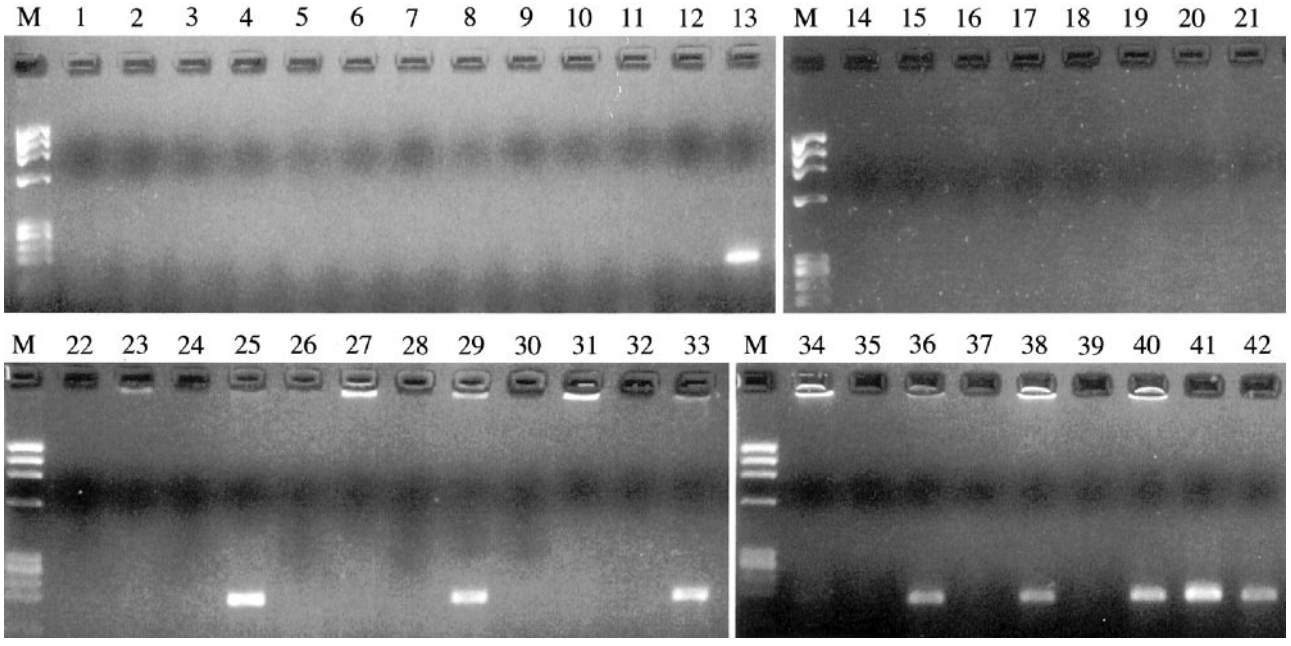


Figure 1. Top: Specificity test of Y primers (**lanes 1** to **21**) on PBL-derived DNA obtained from 20 women (**lanes 1** to **12** and **14** to **21**) and one man (**lane 13**, positive control). **Bottom:** Amplification of fetal Y-chromosomal sequences (198 bp) in single large cells isolated from maternal blood. **Lanes 22** to **42:** Single cells isolated from mothers carrying a male fetus (**lanes 23**, **25**, **27**, **29**, **31**, **33**, **34**, **36**, and **38**). Fetal Y-positive cells: **lanes 25**, **29**, **33**, **34**, **36**, and **38**. Maternal Y-negative cells: **lanes 23**, **27**, **31**. Negative controls: buffer without sample inserted at the cell lysis step and run to the end of the test: **lanes 22**, **24**, **26**, **28**, **30**, **32**, **35**, **37**, and **39**. Positive controls: one single HuH6 cell (**lane 40**), 5 ng and 2 ng of male PBL-derived DNA (**lanes 41** and **42**). M, molecular weight marker (ϕX174 *Hae*III digested).

ward primer 5′-ATTTGATGGAGTTGGACATGGC-3′; reverse primer 5′-ATCAGCTCTTGTTCTTGAGTGA-3′) in 100 μl total volume containing 2 mmol/L $MgCl_2$, 0.25 mmol/L dNTP, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus). Two μl of the PCR product was reamplified with inner primers (forward primer: 5′-ACATGGCCATG-GAACCAGACAGA-3′; reverse primer 5′-GAGTGAAG-GACTGAGAAAATCCCTG-3′). Both PCR tests were performed in a Thermal Cycler (40 cycles: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds). PCR products were purified using spin columns (Microspin Column, Amersham Pharmacia Biotech) and sequenced on ABI Prism 310 (PE Applied Biosystems) using the Big Dye Terminator Cycle Sequencing kit and the two β-catenin inner primers. Mutations were confirmed by sequencing of the two DNA strands from at least two independent PCR products.

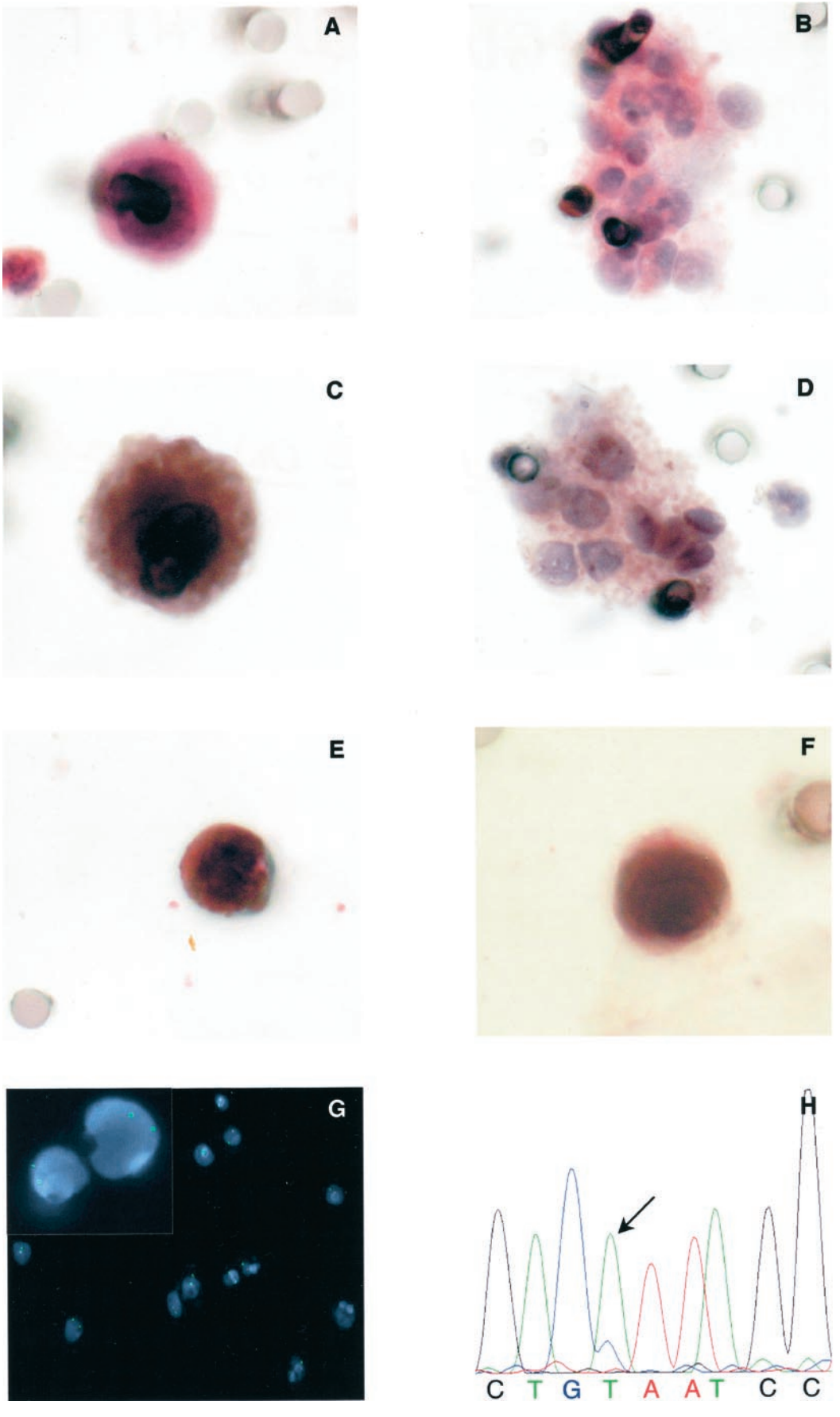
Fluorescence in Situ *Hybridization (FISH) Analysis*

The membranes were pretreated with Triton 0.2% for 10 minutes at room temperature, washed twice in phos-

Table 1. Amplification of Fetal Y-Chromosomal Sequences of Single Cells Isolated from Maternal Blood

Mothers	Microdissected cells	Y-positive cells	Mononucleated cells (Y-positive)*	Polynucleated cells (Y-positive)*
Male fetuses				
1	2	2	1 (1)	1 (1)
2	3	2	3 (2)	0
3	8	7	6 (5)	2 (2)
4	2	1	2 (1)	0
5	3	1	3 (1)	0
6	5	2	5 (2)	0
Total	23	15	20 (12)	3 (3)
Female fetuses				
7	6	0	6	0
8	3	0	1	2
9	3	0	2	1
10	2	0	1	1
11	6	0	5	1
12	3	0	3	0
13	3	0	2	1
Total	26	0	20	6

*The number of Y-positive cells is indicated in parentheses.


A
B
C
D
E
F
G
H
C T G T A A T C C

phate-buffered saline 1× for 2 minutes and once in standard saline citrate 2× for 2 minutes. They were then dehydrated through an ethanol series (70%, 80%, 90%, and 100%) at room temperature and air-dried. Interphase nuclei were denatured in 70% formamide/2× standard saline citrate at 72°C for 4 minutes, dehydrated in ice cold 70%, 80%, 90%, and 100% ethanol and air-dried. Finally, the membranes were treated by Proteinase K (0.5 μg/ml) at 37°C for 22 minutes, dehydrated through an ethanol series, and air-dried. Chromosome X centromeric probe (PDMX1) was directly labeled with green-colored fluorescein-12 dUTP (Roche Molecular Biochemical, Meylan, France) using a nick translation kit (Vysis, Downers Grove, IL) following the supplier's recommendations. Hybridization and analysis were performed according to standard procedures.[11]

Immunohistochemical Characterization of Cells Isolated by ISET

Cells were permeabilized with 0.2% Triton for 10 minutes before immunostaining. Primary antibodies were diluted 1:100 in 10% fetal calf serum and applied to the spot for 1 hour at room temperature. We used KL1 (Cytokeratin gp 56 kd; Immunotech S.A., Marseille, France), a cytokeratin broad-spectrum monoclonal antibody; anti-placental alkaline phosphatase (DAKO, Glostrup, Denmark), a monoclonal antibody for the evaluation of many different types of germ cells; and anti-leukocyte common antigen (DAKO), a monoclonal antibody recognizing a family of high-molecular mass glycoproteins expressed on the surface of the majority of human leukocytes. The spots were then treated as previously described.[8] The following negative controls were performed: 1) the procedure was performed omitting the primary antibody; 2) the primary antibody was substituted by an irrelevant antibody (anti-HPV, B580; DAKO). As positive control, we used fetal cells dissociated from human placenta (gift from Dr. Françoise Ferré, INSERM U361, Paris, France), resuspended in the filtration buffer, and filtered.

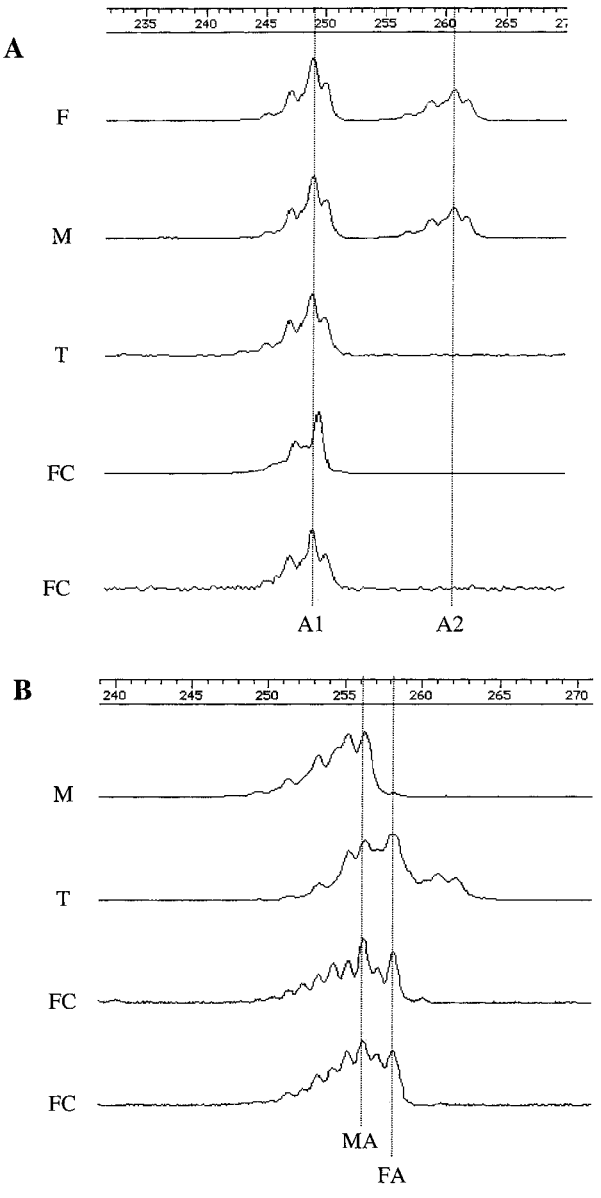


Figure 3. Genotyping by STR markers of parental and trophoblastic DNA and of single-cell DNA isolated from the maternal blood and microdissected. Electrophoretograms of the amplified products obtained using the STR marker D16S3018. **A:** The STR marker does not distinguish paternal (F) from maternal (M) alleles (A1 and A2: 249 and 261 bp). The trophoblastic DNA (T) shows homozygosity for one (249 bp) allele and the same pattern is found in two fetal cells (FC). **B:** The trophoblastic DNA (T) shows the maternal allele (MA: 256 bp), also found in the maternal DNA (M), and the paternal allele (FA: 258 bp). The same two alleles were detected in two microdissected fetal cells (FC).

Results

We assessed the specificity of the Y primers by amplifying the DNA obtained from 20 women. All of the female-derived samples scored negative (Figure 1, top), and the male-derived DNA, used as positive control, scored positive. We then performed a preliminary test on peripheral blood from mothers carrying a male fetus. We microdissected large cells from the corresponding filters and analyzed these cells with Y- and HLA-specific primers. All of the cells scored positive with HLA primers and half of them also scored positive with Y primers. We then went back to the pictures of positive cells and observed two

Figure 2. A to **F:** Morphological and immunohistochemical characterization of circulating cells isolated by ISET and proved to be fetal by Y-specific single-cell PCR testing **A:** Mononucleated cell (diameter, 27.5 μm) with cytotrophoblastic-like morphology laying on a filter pore (round mark in the nucleus). Three empty pores are visible on the **top right** and a neutrophil on the **bottom left** of the picture (H&E staining). **B:** Polynucleated, syncytiotrophoblastic cell (H&E). **C:** Cytotrophoblastic cell positive to the KL1 antibody. **D:** Syncytiotrophoblastic cell positive to the anti-placental alkaline phosphatase antibody. **E** and **F:** Maternal cells positive to the anti-leukocyte common antigen antibody. **G:** FISH analysis performed with a X-specific probe on HuH7 cells mixed with blood and filtered. The majority (~98%) of the filtered cells are labeled. Two cells are shown in the **inset** at higher magnification (×60). **H:** Sequence analysis of β-catenin exon 3 showing the G to T mutation leading to the Gly to Val mutation (codon 34) in a single HuH6 cell. Original magnifications: ×10 (**G**); ×40 (**B** and **D**); ×60 (**A**, **C**, and **E**); ×80 (**F**).

Table 2. STR Genotyping of Single Cells Isolated from Maternal Blood

Mother no.*	Single cell	Y-specific PCR	Single-cell genotyping		
			D16S539 marker	D16S3018 marker	D16S3031 marker
2	2a	+	FC		
	2b	+	FC		
3	3a	+		FC	
	3b	+		FC	
	3c	−			MC
	3d	+			FC
4	4a	−		MC	
	4b	+		FC	
6	6a	−			MC
	6b	−			MC
	6c	−			MC

*See Table 1.
MC, maternal cell profile; FC, fetal cell profile.

types of morphological features (Figure 2, A and B): 1) mononucleated, cytotrophoblast-like cells with a diameter ranging from 14.3 to 30 μm, large nucleus with rather condensed chromatin and small cytoplasm, sometimes with few microvilli at the membrane surface (not shown);[12] and 2) polynucleated, syncytiotrophoblastic cells with a larger diameter (in the range of 44 to 60 μm). We then microdissected 23 large cells with a fetal morphology from the peripheral blood samples of women carrying a male fetus. All these cells scored positive for HLA primers (data not shown) and 15 of them also scored positive for Y-specific primers (Table 1 and Figure 1, bottom). To further test the specificity of our results, we reamplified the Y PCR products with the same Y-specific primers. All of the controls and PCR-negative cells scored negative, whereas positive cells scored positive, and one weakly positive cell (Figure 1, lane 34) exhibited a stronger band (data not shown). Overall, a variable number of cells (from 1 to 7), isolated from 2 ml of blood, was proven to be of fetal origin (Table 1). However, we did not microdissect all of the cells with a fetal-like morphology. Based on morphological data, a preliminary, rough estimation of their mean number was ~5 per ml. To further verify that our approach specifically identifies fetal cells, without false-positive results, we microdissected 26 fetal-like cells isolated from the peripheral blood of seven women carrying a female fetus. All these cells scored positive for HLA primers, whereas none of them scored positive for Y-specific primers (Table 1).

In four cases, paternal blood DNA and/or maternal blood DNA (obtained before pregnancy) and trophoblastic DNA were available. These samples were used to identify circulating fetal cells independently from the fetal gender (Figure 3, Table 2). In one case (Figure 3A), the tested STR marker could not distinguish paternal (F) from maternal (M) alleles (249 and 261 bp), however, the trophoblastic DNA (T) showed homozygosity for one (249 bp) allele. We found the same pattern in two fetal cells (FC), which also scored positive with the Y-specific primers. In another case (Figure 3B), the trophoblastic DNA (T) showed, in addition to the maternal allele (256 bp), also found in the maternal DNA (M), the paternal allele (258 bp). These two alleles were also found in two microdissected cells (FC), therefore demonstrating their fetal genome. By using three STR markers and one-fifth of the single cell DNA, we analyzed 11 microdissected cells isolated from mothers carrying a male fetus and previously tested by using Y-specific primers (Table 2). Six cells revealed a fetal profile and five showed a maternal profile, consistently with Y-specific results.

Taken overall, our results thus provide the molecular proof that we enriched fetal cells and that identification of single fetal cells by this approach is feasible, independently from the fetal gender.

The feasibility of FISH analysis on ISET membranes and of detection of DNA point mutations in single microdissected cells has been provided using cells from cell lines. HuH7 cells were mixed with blood and analyzed by ISET. The FISH protocol with a X-specific probe was then applied to the filter. Results demonstrated a X-specific signal in the majority (98%) of the filtered cells (Figure 2G). HuH6 cells, known to carry a point mutation in the β-catenin gene (codon 34: GGA to GTA, Gly to Val mutation)[13] were mixed with blood and individually microdissected. The β-catenin exon 3 was then amplified by a nested protocol and the PCR product was analyzed by sequencing. The results of this test consistently showed a point mutation at codon 34 (G to T) in five analyses independently performed on five different HuH6 cells (Figure 2H). These results demonstrate that the ISET approach can be combined to the FISH analysis for prenatal diagnosis of chromosomal abnormalities. They also show the feasibility of DNA point mutation detection in single microdissected cells.

To characterize some large cells isolated by ISET we performed immunohistochemical analyses on peripheral blood derived from mothers carrying a male fetus. After immunohistochemistry, large cells were microdissected and tested by PCR with Y-specific primers. Fetal mononuclear cells and syncytiotrophoblastic cells appeared to be positive to the KL1 antibody or to the anti-placental alkaline phosphatase antibody (Figure 2, C and D). Maternal cells were positive for anti-leukocyte common antigen antibody (Figure 2, E and F).

Discussion

This study describes a new approach allowing the highly efficient enrichment of fetal cells circulating in the maternal blood. In fact, fetal cells were found in the blood samples from all of the tested mothers, by analyzing only 2 ml of peripheral blood. A clear advantage of ISET,[8] a highly sensitive tool for the enrichment of epithelial cells, over flow cytometry and immunomagnetic cell selection, is that both damage and loss of fetal cells are minimized. This powerful enrichment, when combined with single cell microdissection, enables fetal cell identification by single-cell DNA amplification of Y sequences and/or highly polymorphic STR markers. As previously pointed out,[14] the clinical impact of the STR strategy, which allows identification of fetal cells independently from the fetal gender, is dependent on an efficient approach for fetal cell enrichment and microdissection. Our work provides an example of this successful combination.

Fetal and nonfetal large cells circulating in the peripheral blood and isolated by ISET have been characterized by cytomorphological studies and by immunohistochemistry. Fetal large cells include mononucleated cells, which are mainly cytotrophoblastic cells, because they score positive with the KL1 antibody and syncytiotrophoblastic cells, with a typical morphology of polynucleated cells.[15] Nonfetal large cells include lymphoid and/or myeloid cells that scored positive with the anti-leukocyte common antigen antibody. Although this analysis is interesting, it is noteworthy that only the identification of fetal cells by genetic analysis can be considered certain enough to allow prenatal diagnostic tests. Lymphoid and/or myeloid fetal progenitors, but not trophoblastic cells, have been shown to persist postpartum in the maternal blood.[16] Therefore, microdissection of trophoblastic cells enables the focusing of genetic tests on the ongoing pregnancy.

Another fundamental aspect of this approach is the possibility of performing at least five PCR analyses starting from the DNA of one individual cell. This allows to perform genetic testings selectively on cells proved to be fetal by DNA analysis. Fetal cells can now be identified by STR polymorphic markers[17,18] and the genetic diagnosis can be based reliably on the DNA analysis of several individual fetal cells.

The prenatal detection, using FISH, of fetal cells with three chromosome-21 signals in the maternal plasma has recently been reported.[19] This approach is also interesting, however fetal cells are found only rarely in plasma (1 in 500 to 1 in 2000) and are mainly apoptotic cells[20] and hence not the best target for genetic analyses. Furthermore, euploid fetal cells from female fetuses cannot be identified by this approach. In this context, we show here that the FISH protocol can be successfully applied to cells enriched by ISET. FISH can therefore be combined with ISET as a first screening for prenatal aneuploid disorders. Single cell microdissection and specific genetic analyses (single cell CGH and/or quantitative allelic studies) focused on genetically proved fetal cells then would be performed.

A proportion of fetal DNA (~3.4% of maternal DNA)[21] is also present in the maternal plasma fraction and is accessible for genetic testing. Although this fraction is useful for assays such as gender testing and detection of RHD sequences,[22,23] the mixing of fetal and maternal DNA may hamper detection of fetal DNA point mutations and quantitative allelic studies.

Our approach provides evidence that the target cells do not have apoptotic morphology, and that genetic analyses may be directed toward a pure fetal cell genome. In this context, we have shown that reproducible detection of point mutations (β-catenin exon 3 mutation) is feasible by combining ISET and single cell microdissection.

In conclusion, we have described a reliable approach, based on single cell genetic characterization of highly enriched fetal cells, that may have implications for noninvasive prenatal diagnosis of genetic disorders.

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