DOI: 10.1002/pd.4429

ORIGINAL ARTICLE

A new marker set that identifies fetal cells in maternal circulation with high specificity

Lotte Hatt[1,4], Marie Brinch[1,4], Ripudaman Singh[1,4], Kristine Møller[1,3], Rune Hoff Lauridsen[1,4], Jacob Mørup Schlütter[2], Niels Uldbjerg[2], Britta Christensen[1,4] and Steen Kølvraa[1,4]*

[1]Department of Clinical Genetics, Vejle Hospital, University of Southern Denmark, Vejle, Denmark
[2]Department of Obstetrics and Gynecology, Aarhus University Hospital, Skejby, Aarhus, Denmark
[3]Department of Physiotherapy and Occupational Therapy, Aarhus University Hospital, Aarhus, Denmark
[4]FCMB ApS, Vejle, Denmark
*Correspondence to: Steen Kølvraa. E-mail: Steen.Koelvraa@slb.regionsyddanmark.dk

ABSTRACT

Objective Fetal cells from the maternal circulation (FCMBs) have the potential to replace cells from amniotic fluid or chorionic villi in a diagnosis of common chromosomal aneuploidies. Good markers for enrichment and identification are lacking.

Method Blood samples from 78 normal pregnancies were used for testing the marker-set CD105 and CD141 for fetal cell enrichment. Fetal cell candidates were subsequently stained by a cocktail of cytokeratin antibodies, and the gender of the fetal cells was explored by fluorescence *in situ* hybridization (FISH) of the X and Y chromosomes.

Results Fetal cell candidates could be detected in 91% of the samples, and in 85% of the samples, it was possible to obtain X and Y chromosomal FISH results for gender determination. The concordance between gender determined by FISH on fetal cells in maternal blood and gender found at birth reached 100% if three or more fetal cells with FISH signals could be found in a sample.

Conclusion The marker set identifies fetal cells with specificity high enough to make cell-based noninvasive prenatal diagnosis realistic.

Funding sources: The experiments described in this article were funded by the private company FCMB ApS. The ultimate goal of FCMB ApS was to develop a new method for noninvasive prenatal diagnostics based on identification of fetal cells in the maternal circulation.
Conflicts of interest: The experiments described in this article were performed in the private company FCMB ApS in which the authors MB, RS, LH, KM, RHL, and BC were employed. SK and BC are founders of this company. The authors MB, RS, LH, BC, and SK have filed patent applications on the isolation and identification of fetal cells in maternal blood for noninvasive prenatal diagnostics.

INTRODUCTION

Noninvasive prenatal diagnosis (NIPD) has long explored the potential use of fetal cells in the maternal circulation. These very rare fetal-originated cells have proved difficult to find with consistency in blood samples from pregnant women, both due to their rarity, and also due to the lack of knowledge on the exact fetal cell type present in maternal blood. Because of this lack of knowledge on the fetal cell type, several possible cell types, ranging from lymphoblasts, erythroblasts to trophoblasts have been investigated.[1–5] However, no specific fetal cell markers optimal for enrichment and subsequent immunohistochemical identification have yet been identified.

For several years, we have therefore sought to identify one or more fetal cell type in the maternal blood circulation in order to define specific fetal cell markers that can be used for developing a cell-based, noninvasive prenatal diagnostic method. In this research line, we chose to make cDNA expression libraries of fetal cells isolated from the maternal circulation.[6,7] We thereby demonstrated that a group of fetal cells expressed both ectodermal markers and endothelial vascular markers. This marker combination made us conclude that these cells most likely belonged to a subgroup of extravillous trophoblast (EVTs) named endovascular. These endovascular EVTs migrate into the placental bed where they reach the maternal arteries and here replace the maternal endothelial cells during early pregnancy, thereby ensuring sufficient blood flow to the placenta and fetus.[8,9] The endothelial/vascular marker expression of these originally ectodermal cells is the result of an adaptation to the new vascular environment.[10]

The combination of ectodermal and endodermal markers on the same cell type is rarely observed in blood, and we therefore hypothesized that this combination could be a useful target for specific fetal cell enrichment and identification. In the present

study, we isolated fetal cells from blood samples from pregnant woman (gestational age 11–13 weeks) using magnetic cell sorting enrichment [magnetic-activated cell sorting (MACS)] with a novel antibody combination for the endothelial/vascular markers CD105 and CD141. Isolated cells were subsequently stained with a cocktail of different ectodermal cytokeratin antibodies. In a series of 78 normal pregnancies, gender was determined by fluorescence *in situ* hybridization (FISH) of these cytokeratin-positive cells, and the findings were correlated with the gender revealed at birth. We find that CD105/CD141 enrichment combined with positive cytokeratin staining is a unique marker combination that results in a highly specific identification of fetal cells in maternal blood. The method has the potential to be used in future noninvasive prenatal diagnostics.

MATERIALS AND METHODS

Patient material

All participants were recruited when they attended the first trimester nuchal translucency scanning session at Aarhus University Hospital in 2011. Ninety pregnant women, gestational age 11–14 weeks, were included. For identification of fetal cells in maternal blood, 30 mL blood was drawn in heparin-containing tubes. All participants signed informed consent. The true gender of the fetus was obtained from patient files of the mothers after delivery. The project was approved by the local Danish Scientific Ethical Committee (S-20070045) and the Danish Data Protection Agency (2008-58-0035).

Blood preparation and fetal cell enrichment

All tubes and pipette tips used in this procedure were precoated with a precoating buffer (2% BSA in PBS w/o Ca^{2+} and Mg^{2+}) to minimize cell loss.

Whole blood fixation, red blood cell lysis, and cell permeabilization

A total of 30 mL of heparinized blood samples were fixed within 15 min of drawing in 4% formaldehyde as described in the work by Hatt *et al.*[7] We used the same technique as previously published[7] for lysis of red blood cells and permeabilization of the nucleated cell. The final cell pellet was resuspended in 280 μL MACS buffer (phosphate-buffered saline pH 7.2, 0.5% bovine serum albumin, and 2 mM ethylenediaminetetraacetic acid).

MACS

Enrichment of CD105/CD141-positive cells was performed using three different kinds of primary antibodies mixed together according to the manufacturer's (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) basic protocols with slight modifications. Since, we have previously shown that the CD105 is responsible for the enrichment of the majority of the fetal cells, we chose to add an extra monoclonal antibody against CD105. In this study, 163 μL of CD105 microbeads (Miltenyi, no.: 130-051-201), 163 μL CD141 microbeads (Miltenyi, no.: 130-090-521), 163 μL FcR blocking reagent (provided with the microbead kits), and 30 μL (15 μg) monoclonal anti-CD105 antibody (eBioscience, no.: 14-1057) were added to the 280 μL cell suspension. The cell suspension was incubated for 60 min at 4 °C, washed twice with 14 mL MACS buffer (4 °C), recovered by centrifugation for 10 min at 500 g, and resuspended in 600 μL MACS buffer (4 °C). Then, 150 μL of goat anti-mouse IgG microbeads (Miltenyi, no.: 130-048-401) were added. The cell suspension was incubated for 30 min at 4 °C, washed twice with 14 mL MACS buffer (4 °C), recovered by centrifugation for 10 min at 500 g, and resuspended in 2 mL MACS buffer (4 °C). The cell suspension was added to two prewashed MS columns (Miltenyi, no.: 130-042-201) that were stacked on top of two other prewashed MS columns. To wash the columns, 3 × 500 μL MACS buffer (4 °C) was added to each of the top MS columns. The four MS columns were then removed from the magnet, placed on a precoated 15 mL collection tube, and the cells were eluted by applying 1 mL of cold MACS buffer twice to each column. The first milliliter of buffer ran through the column by gravity force. The second milliliter of buffer was forced through the column by applying a plunger. The collection tube was centrifuged at 500 g for 10 min, and the cell pellet was resuspended in MACS buffer (4 °C). The cell suspension was placed on FLEX IHC slides (DAKO) in 40 μL aliquots (approximately 50 000 cells per slide), and the slides were air-dried overnight.

Immunostaining, automated scanning, and FISH

Antibody staining

Antibody staining were performed on an autostainer (DAKO, DK). Microscope slides were incubated for 7 min on a 60 °C hot plate, fixed for 10 min in 2% formaldehyde in PBS, washed for 5 min in PBS and 10 min in 4xSSC/0.1% tween 20. The slides were then loaded into the DAKO autostainer, rinsed once with 4xSSC/0.1% tween 20, and incubated for 30 min with 300 μL Image-iT FX signal enhancer (Invitrogen). After one rinse in 4xSSC/0.1% tween 20, the slides were incubated for 30 min with blocking buffer (4xSSC, 0.5% blocking reagent (Boeringer), 1% BSA, 10% goat serum) followed by 30 min with 300 μL of a mixture of anti-cytokeratin 7 (DAKO), anti-pan cytokeratin (Sigma), and anti-cytokeratin 8/18 (Invitrogen) all diluted 1 : 100 in blocking buffer. Slides were then rinsed four times with 4xSSC/0.1% tween 20 and incubated for 30 min with 300 μL Alexa Fluor 488 rabbit anti-mouse IgG (component A, Invitrogen) diluted 1 : 200 in blocking buffer. After four times rinse in 4xSSC/0.1% tween 20, slides were incubated for 30 min with 300 μL of Alexa Fluor 488 goat anti-rabbit IgG (component B, Invitrogen) diluted 1 : 200 in blocking buffer. Slides were subsequently rinsed thrice in 4xSSC/0.1% tween 20, once in 2xSSC, fixed for 10 min in 2% formaldehyde in PBS, washed for 5 min in 2xSSC, and mounted in Vectashield with 0.6 μg/mL DAPI (Vector Laboratories).

Automated scanning

Cytokeratin-stained cells were identified by automatic scanning of slides using the MetaCyte scanning program developed by Metasystems. Slides were scanned at 10× magnification using a classifier developed and optimized in-house.

Manual validation of stained cells

Green-fluorescent cells picked out by the microscope scanners were finally validated manually based on the general appearance of epithelial cells. We classified cells as fetal candidates if they had a strong homogenous staining pattern or if they had a clear 'bubbled' staining; a sign of actin degradation during mesenchymal/epithelial transition or apoptosis. Furthermore, a stained cell should have a cytoplasm clearly larger than the nucleus and elongated or irregular shape to be classified as a fetal cell candidate.

Gender determination by X-chromosome and Y-chromosome-specific FISH

Before FISH, cover slips were removed by washing the slides for 15 min in 2% formaldehyde in PBS. The slides were fixed for 10 min in 4% formaldehyde in PBS, dehydrated through 60%, 80%, and 99.9% ethanol and air-dried. X-chromosome and Y-chromosome FISH was performed as described in Christensen *et al.*[2] Chromosome-specific repeat probes, DXZ1 (CEP X alpha satellite) labeled with spectrum aqua and DYZ1 (CEP Y satellite III) labeled with spectrum orange (Abbott Molecular) were used for hybridization. After FISH, slides were mounted in Vectashield with 0.6 µg/mL DAPI (Vector Laboratories).

The slides were placed in the scanning microscopes, candidate fetal cells were relocated, and the number of X and Y chromosome signals in these cells was counted manually.

RESULTS

Blood sample processing for retrieval of candidate fetal cells – removal of maternal blood cells and staining of candidate fetal cells

To stabilize fetal cells and diminish apoptosis and lysis of these rare cells, blood samples were fixed with formaldehyde within 15 min of blood withdrawal and before selective lysis of red blood cells. To remove the bulk of maternal white blood cells, we subsequently applied MACS with antibodies against the vascular surface protein markers CD105 and CD141 markers, which, as demonstrated in previous studies, are capable of enriching fetal cells from maternal blood samples[7] (and unpublished results). Seventy-eight of the initial 90 blood samples were processed in this way. Twelve samples were discarded during the enrichment procedure because of problems regarding clogging of the MACS columns (see Figure 1 for an overview of the blood samples). These problems most likely occurred because the MACS columns used were not designed for fixed cells, which are more prone to stick to the column-material than unfixed cells. For the samples that did not clog the column, the combination of red blood cell lysis and MACS vastly reduced the number of maternal cells (Table 1) and left us with a manageable number of cells for antibody staining and automatic microscope scanning.

To specifically stain fetal cells, we used antibodies against cytokeratins, which are typical epithelial cell markers. Automatic microscope scanning for stained cells produced around 5000 hits per sample, which were subsequently inspected. Most of these 5000 hits were clear artifacts with no similarity with cells, and they could be discarded at a quick glance. In addition to truly stained cells, we also identified a number of cells entirely covered with weak green unspecific staining and cells with apparent green dirt particles on their surface. Judging by the brightness and pattern of the staining, a small number of cells (0–18 cells per sample) were classified as candidate fetal cells.

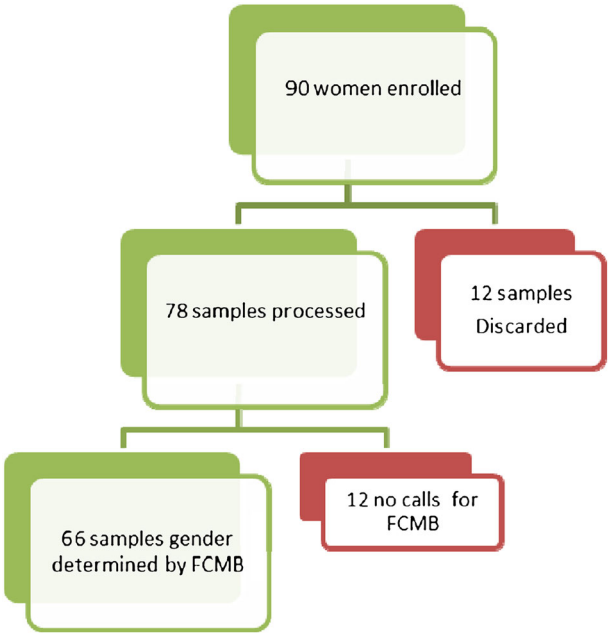

Figure 1 Samples in numbers. Blood samples were drawn from 90 pregnant women, of which 78 were correctly processed and analyzed. Twelve samples were discarded because they clogged the column. In total, 66 of the 78 processed samples could be gender determined by FISH of FCMBs. Twelve of the 78 samples did not contain any FISH signals (no calls)

Table 1 Reduction of maternal white blood cells

Method	Maternal white blood cells in a 30 mL blood sample
Whole blood	1.5×10^8
After positive selection by MACS	500 000
After scanning for stained cells	5000
After manual classification of fetal cells	0–1

MACS, magnetic cell sorting enrichment.

Estimated reduction of maternal white blood cells during the process of fetal cell enrichment and staining of a 30 mL blood sample. The calculation was based on an estimated value of 5 × 106 WBCs per 1 mL of whole blood. After positive selection the cell numbers were estimated by several cell counts resulting in an approximate average of 500 000 cells. The automated scanning of the cytokeratin stained cell-slides resulted in around 5000 hits that had to be checked manually in the microscope. The manual validation resulted in extremely few maternal cells.

FISH-based gender determination of individual candidate fetal cells

In total, 334 cytokeratin-stained cells were identified in 78 blood samples. The candidate fetal cells identified by cytokeratin staining were subjected to FISH with a red Y-chromosome probe and a blue X-chromosome probe in order to determine the gender of the fetus based on circulating fetal cells. Cells were classified as male cells if they contained one

red and one blue FISH signal, whereas female cells should contain two blue FISH signals. An example of a stained male fetal cell before and after FISH is shown in Figure 2.

Details of the FISH results are shown in Table 2. In total, 15% of the 334 fetal cell candidates were lost during the FISH procedure, and 9% had no detectable FISH signal. Clear FISH signals were seen in the remaining 255 of the cells, most of which were identified as XX (38%) or XY cells (55%), but there was also a small number of X0, Y0, XYY, XXX, and XXXX cells (8%). We only employed XX and XY cells for the FISH-based gender determination.

Only candidate fetal cells from women carrying a male fetus can be used to determine the specificity of cytokeratin antibodies for identification of true fetal cells in the enriched cell fractions, because these candidate fetal cells can be verified by the presence of a Y chromosome. In a blood sample from a woman carrying a female fetus, a true (XX, female) fetal cell cannot be distinguished from false positive (XX) maternal cells. In 34 samples from pregnant women verified to be carrying male fetuses, a total of 145 green stained putative fetal cells were identified. Among these, six cells contained two X signals instead of an X and a Y signal. They were in all probability stained maternal cells. Hence, the fetal cell specificity of the cytokeratin stain on the enriched population of cells containing FISH signals is 96% (Table 3).

In 66 of the 78 processed samples, we identified at least one fetal cell with FISH signals, and these samples were subsequently used to predict the fetal gender. For one of these 66 samples, the patient had relocated to another hospital, so the gender at birth could not be obtained, leaving us with 65 samples. By this method, 32 fetuses were classified as male (XY) and 33 as female (XX). For two of these samples, the gender was incorrect, which resulted in a gender concordance of 97.0% if all samples with fetal cells containing FISH signals were included without regard to how few fetal cells were present in individual samples (Table 4). In the remaining 12 samples, we found no candidate

Table 3 **Specificity of identification of fetal cells by cytokeratin staining**

	CK pos cells with FISH signal	XX cells by FISH	XY cells by FISH	Specificity of the CK stain
Male fetus samples ($n = 34$)	145	6	139	96%
Female fetus samples ($n = 31$)	87	87	0	—

FISH, fluorescence *in situ* hybridization.
This table shows gender determination by FISH of fetal cell candidates stained by the cytokeratin antibodies in pregnancies with male and female fetuses, respectively. Of the 145 cells from pregnancies carrying a male fetus, six cells contained two X chromosome signals (XX) indicating maternal origin. The remaining 139 fetal candidate cells all exhibited one X chromosome and one Y chromosome FISH signal. Hence, the specificity of the CK antibodies for true fetal cells is 96%. The specificity cannot be calculated for the female fetus pregnancy samples because it is not possible to distinguish between true female fetal cells and maternal blood cells.

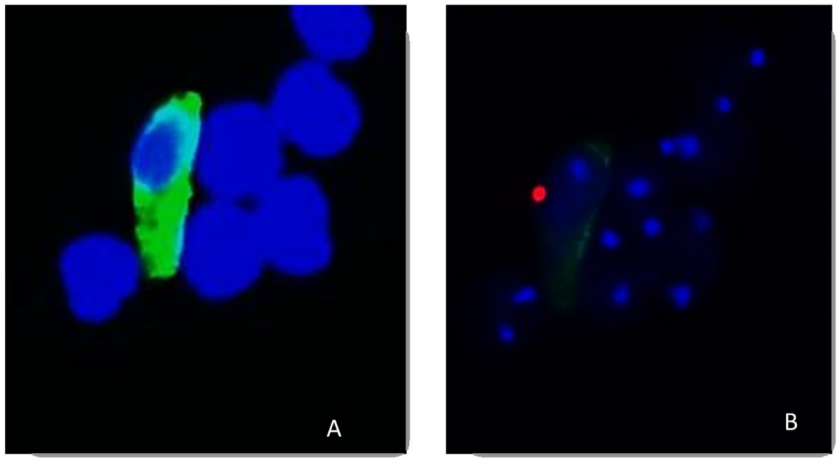

Figure 2 **Fetal cell staining. Picture (a) shows a fetal cell stained green with cytokeratin antibodies. Nuclei are stained blue with DAPI. Picture (b) shows the same fetal cell after fluorescence in situ hybridization (FISH) with a red Y-chromosome probe and a blue X-chromosome probe. The fetal cell originates from a male fetus having one red Y and one blue X signal. The surrounding cells are maternal each containing two blue X signals**

Table 2 **Fluorescence *in situ* hybridization results on CK positive cells in exact numbers**

Samples	CK positive cells	XX	XY	X0	Y0	XYY	XXX	XXXX	Cell lost	No signal	Cytokeratin positive cells with FISH signals
78	334	96	139	3	5	1	10	1	50	29	255

FISH, fluorescence *in situ* hybridization.
Distribution of the FISH results on cytokeratin (CK) stained cell. From the 78 processed maternal blood samples, a total of 334 fetal cell candidates were stained with the CK antibodies. All 334 cells underwent FISH. In total, 255 of the 334 cells contained clear FISH signals. During the FISH procedure, 50 cells were lost (fell of the slide), and another 29 cells did not contain any FISH signals. The distribution of cells containing XY, XX, or numerical aberrant FISH signals (XO, YO, XYY, and XXX) can be seen in the table.

Table 4 Concordance between gender diagnosed by fetal cells from the maternal circulation and the true gender given at birth

Method for fetal gender determination or identification	XX	XY	No call	Correct gender	Incorrect gender	% Concordance with gender at birth
FCMB	33	32	12	63	2 XX	97%
Birth	39	38	1*	77		

FCMB, fetal cells from the maternal circulation.

This table shows fetal gender determination by FCMBs compared with the correct gender of the baby given at birth. In 66 of the 78 processed blood samples, we found at least one stained fetal cell candidates with clear sex-chromosome FISH signals. However, one of the participants moved during the study, so for this patient, the gender was classified as a no call (1*), leaving 65 samples for comparison for gender concordance. Two of the 65 samples were incorrectly classified by FCMBs as female fetus pregnancies thereby giving a concordance of 97% compared with the true fetal gender when all 65 samples were included.

fetal cells or the candidate fetal cells were lost during the FISH procedure. Thus, for these 12 samples, we could not determine the gender by FISH, and they were classified as 'no calls'.

Table 5 presents the relationship between correct gender determination by FISH and number of candidate fetal cells available for each sample. As seen here, it requires at least three fetal cells to obtain 100% concordance between the FISH-based gender determination and the true gender at birth.

A detailed presentation of the distribution of fetal cell numbers in the various samples can be seen in Figure 3. The slightly different distribution seen before and after FISH is firstly due to the fact that a small fraction of the fetal cells were lost from the microscope slides during the FISH procedure and secondly that a fraction of the fetal cells contained no FISH signal. The average number of stained fetal cells per sample was 4.3 with a distribution of 0–18 cells per sample, whereas the average number of fetal cells with FISH signals was 3.3 with a distribution of 0–15 cells per sample.

DISCUSSION

In our previous studies, we suggested that some of the fetal cells in the maternal circulation are EVTs.[6,7] This suggestion was based on the fact that gene expression profiles of fetal cells enriched from maternal blood by CD105 antibody revealed that some of the fetal cells had a unique combination of endothelial and ectodermal markers. From this, we found that CD105 and CD141 together with cytokeratin was a marker set that detected fetal cells in maternal blood.

The aim of the present study was to more precisely evaluate this marker set for fetal cell detection in maternal blood. This was accomplished by determining the gender of fetuses based on fetal candidate cells found in blood from a large group of pregnant women coming for a 12-week nuchal translucency scanning and comparing the results with the gender found after birth.

As a first step, we demonstrated an excellent fetal cell specificity of the cytokeratin staining in the enriched cell fractions reaching 96%. This specificity was calculated from a relatively large pool of 145 fetal cells from pregnancies with male fetuses. For the FISH-based fetal gender determination, we started out with 78 samples. Of these, we found fetal cells in 91%, and we obtained clear FISH results on fetal cells in 85% of the processed samples. These samples allowed us to estimate the fetal gender based on the circulating fetal cells. By comparing these data with the gender found at birth, we found a 100% concordance if at least three fetal cells with two FISH signals were found in a sample. These findings clearly indicate that the cells detected by the marker set represent a specific group of fetal cells that could probably be used for cell-based NIPD.

We did, however, encounter a relatively large cell loss throughout the enrichment procedure. In total, 13% of the samples clogged the MACS columns, which forced us to discard the samples. We know from communication with Miltenyie Biotech that prefixed cells are not optimal for use in the MACS column because the cells have a tendency to become sticky and can therefore clog the columns. On the other hand, we have observed that a quick fixation results in

Table 5 Fetal gender concordance in relation to number of fetal cells per sample

Number of fetal cells/sample	0 cells	1–2 cells	3–5 cells	>5 cells
Gender concordance to gender at birth	No call	94%	100%	100%

This table shows concordance between the fetal gender determined on FCMBs by FISH compared with the true gender identified at birth when split into groups with regards to fetal cell number/sample. If no fetal cell candidates contained conclusive FISH signals, the sample was classified as no call. Fetal gender determination based on only one to two fetal candidate cells per sample results in 94% fetal gender concordance. When looking at samples with three or more fetal candidate cells, a correct fetal gender determination was obtained in all samples.

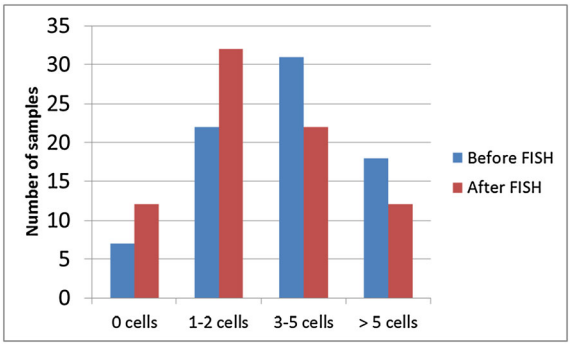

Figure 3 Distribution of 78 samples according to the number of fetal cells per sample. Distribution of 78 samples according to the number of fetal cells per sample. The blue columns show the distributions of samples according to the number of cytokeratin stained fetal candidate cells before FISH and the red columns show the distribution of samples according to the number of fetal cells confirmed by FISH

a larger fetal cell number (data not shown). Most likely, the quick fixation stabilizes the fetal cells which would otherwise be fragile.

Analyzing the fetal cell gender by X and Y chromosome FISH revealed that a number of cells (9%) could not obtain FISH signals. We also observed that a fraction (12%) of the male fetal cells had a fragmented Y-signal but an intact X signal. We believe that this could be due to incipient degradation of the chromosomes, which is probably induced by apoptosis. The X-probe is a centromeric probe, whereas the Y-probe is locus-specific probe located on the chromosome arm, which makes it an earlier target for the DNA fragmentation, which starts at the distal telomeric end of the chromosomes.

It was a concern that 15% of the fetal cells were lost from the slides during the FISH procedure. We speculate that these cells are among the most fragile of the fetal cell and therefore cannot withstand the relative harsh FISH treatment. Another possibility is that cells are simply torn of the slide when we remove the coverslips of the slide.

Because we have only 8% numerical aberrations in our chromosomal FISH signal, which is close to the range of normally reported FISH errors, we do not believe that chromosome instable cells such as syncytiotrophoblast cells form any significant fraction of the fetal cells found in this study.

The use of trophoblasts as a target for NIPD has been pursued before. Van Wijk *et al.* searched for EVTs in a series of 21 disomic pregnancies using density gradient enrichment with a following HLA-G-staining as a fetal-specific marker.[11,12] However, they encountered problems with considerable unspecific HLA-G-staining of maternal cells and low fetal cell numbers. In line with this, Kirszenbaum reported that HLA-G are expressed in some maternal cell, although at low levels, which probably also makes HLA-G a less suitable fetal cell marker.[13] Attempts have also previously been made to use the marker CD105 for isolation of circulating fetal cells in maternal blood, but this attempt also met with limited success.[14,15] These studies were, however, conducted on unfixed Ficoll-Paque™ isolated mononuclear blood cells, a method we have also tried, but without success (data not shown).

It is very likely that the fetal cells found by Mouawia *et al.*[16] are the same subpopulation of fetal cells as those we analyzed. These circulating fetal trophoblastic cells were isolated by size, laser-microdissected, and checked for short tandem repeats before they were analyzed for cystic fibrosis and spinal muscular atrophy.

The ultimate goal for this study is partly to find sufficient fetal cells for FISH-based demonstration of aneuploidies but more important to find enough fetal cells to generate representative genomic DNA from the fetus through whole genome amplification. Because of the extreme rarity of fetal cells in maternal blood, it is at the present time, however, unrealistic to achieve a pure sample of fetal DNA. It is therefore highly relevant that equipments for capturing of individual cells from a cell sample are being developed at this time – to a large extent because of the promises of circulating tumor cells. We therefore envision that using these equipments, fetal cells enriched and stained with our marker combination can be isolated in pure preparations, and the genomic material of these cells can subsequently be amplified. Access to such amplified fetal DNA will make it possible to screen prenatally for minor chromosomal imbalances and combinations of copy number variations with an extremely high resolution. Such a technique could in the future make it possible to NIPD of a much broader range of disorders.

ACKNOWLEDGEMENTS

The authors would like to thank Helle Kristensen, Maja Kristensen, Trine Petersen, Marianne Rasmussen, and Simon Tabi Arrey for expert technical assistance.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Optimal markers for enrichment and detection of sufficient numbers of fetal cells from maternal blood are lacking if fetal cells are to be used in noninvasive prenatal diagnosis.
- Fetal extravillous trophoblasts (EVTs) are present in the maternal blood circulation, but enrichment and specific identification have been suboptimal.

WHAT DOES THIS STUDY ADD?

- This study presents a new marker set for enrichment and identification of fetal EVT cells.
- Fetal EVT cells can be detected with a high specificity of 96%.

REFERENCES

1. Bianchi DW, Simpson JL, Jackson LG, *et al.* Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. Prenat Diagn 2002;22:609–15.
2. Christensen B, Kolvraa S, Lykke-Hansen L, *et al.* Studies on the isolation and identification of fetal nucleated red blood cells in the circulation of pregnant women before and after chorion villus sampling. Fetal Diagn Ther 2003;18:376–84.
3. Guetta E, Gordon D, Simchen MJ, *et al.* Hematopoietic progenitor cells as targets for non-invasive prenatal diagnosis: detection of fetal CD34+ cells and assessment of post-delivery persistence in the maternal circulation. Blood Cells Mol Dis 2003;30:13–21.
4. Krabchi K, Gros-Louis F, Yan J, *et al.* Quantification of all fetal nucleated cells in maternal blood between the 18th and 22nd weeks of pregnancy using molecular cytogenetic techniques. Clin Genet 2001;60:145–50.
5. Oudejans CB, Tjoa ML, Westerman BA, *et al.* Circulating trophoblast in maternal blood. Prenat Diagn 2003;23:111–6.
6. Brinch M, Hatt L, Singh R, *et al.* Identification of circulating fetal cell markers by microarray analysis. Prenat Diagn 2012;32:742–51.
7. Hatt L, Brinch M, Singh R, *et al.* Characterization of fetal cells from the maternal circulation by microarray gene expression analysis – could the extravillous trophoblasts be a target for future cell-based non-invasive prenatal diagnosis. Fetal Diagn Ther 2013.
8. Harris LK. Review: trophoblast-vascular cell interactions in early pregnancy: how to remodel a vessel. Placenta 2010;31 Suppl:S93–8.

9. Kaufmann P, Black S, Huppertz B. Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 2003;69:1–7.
10. Zhou Y, Fisher SJ, Janatpour M, *et al.* Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J Clin Invest 1997;99:2139–51.
11. van Wijk IJ, de Hoon AC, Jurhawan R, *et al.* Detection of apoptotic fetal cells in plasma of pregnant women. Clin Chem 2000;46:729–31.
12. van Wijk IJ, Griffioen S, Tjoa ML, *et al.* HLA-G expression in trophoblast cells circulating in maternal peripheral blood during early pregnancy. Am J Obstet Gynecol 2001;184:991–7.
13. Kirszenbaum M, Moreau P, Gluckman E, *et al.* An alternatively spliced form of HLA-G mRNA in human trophoblasts and evidence for the presence of HLA-G transcript in adult lymphocytes. Proc Natl Acad Sci U S A 1994;91:4209–13.
14. Gussin HA, Sharma AK, Elias S. Culture of endothelial cells isolated from maternal blood using anti-CD105 and CD133. Prenat Diagn 2004;24:189–93.
15. Gussin HA, Sharma AK, Elias S. Culture of cells from maternal circulation, in conditions favoring fetal endothelial cell expansion, does not facilitate the preferential expansion of circulating fetal cells. Fetal Diagn Ther 2005;20:64–9.
16. Mouawia H, Saker A, Jais JP, *et al.* Circulating trophoblastic cells provide genetic diagnosis in 63 fetuses at risk for cystic fibrosis or spinal muscular atrophy. Reprod Biomed Online 2012;25:508–20.