ORIGINAL ARTICLE

Identification of circulating fetal cell markers by microarray analysis

Marie Brinch[1], Lotte Hatt[1], Ripudaman Singh[1], Kristine Møller[1], Steffen Sommer[2], Niels Uldbjerg[3], Britta Christensen[1] and Steen Kølvraa[4]*

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

ABSTRACT

Objective Different fetal cell types have been found in the maternal blood during pregnancy in the past, but fetal cells are scarce, and the proportions of the different cell types are unclear. The objective of the present study was to identify specific fetal cell markers from fetal cells found in the maternal blood circulation at the end of the first trimester.

Method Twenty-three fetal cells were isolated from maternal blood by removing the red blood cells by lysis or combining this with removal of large proportions of maternal white blood cells by magnetic-activated cell sorting. Fetal cells identified by XY fluorescence *in situ* hybridization and confirmed by reverse-color fluorescence *in situ* hybridization were shot off microscope slides by laser capture microdissection. The expression pattern of a subset of expressed genes was compared between fetal cells and maternal blood cells using stem cell microarray analysis.

Results Twenty-eight genes were identified as fetal cell marker candidates.

Conclusion Of the 28 fetal marker candidate genes, five coded for proteins, which are located on the outer surface of the cell membrane and not expressed in blood. The protein product of these five genes, MMP14, MCAM, KCNQ4, CLDN6, and F3, may be used as markers for fetal cell enrichment. © 2012 John Wiley & Sons, Ltd.

Funding sources: The experiments described in this article were performed in and funded by the private company FCMB ApS, in which the authors MB, RS, LH, KM and BC were employed. SK and BC are founders of this company. The ultimate goal of FCMB ApS was to develop a new method for non-invasive prenatal diagnostics based on isolating fetal cells from the maternal circulation. The authors MB, RS, LH, BC and SK have filed patent applications on the isolation and identification of fetal cells in maternal blood for non-invasive prenatal diagnostics.
Conflicts of interest: None declared

INTRODUCTION

It has been known for decades that fetal cells during most of pregnancy pass over in the maternal circulation, and it has long been an ambition to replace invasive prenatal diagnostic procedures such as amniocentesis and chorionic villus sampling with analysis of these cells. It has been envisioned that such a non-invasive prenatal test could be based on isolation of the fetal cells using a cell-type-specific antibody followed by molecular diagnoses of the isolated cells, either by fluorescence *in situ* hybridization (FISH) or by ultrasensitive polymerase chain reaction (PCR).

Although many research groups have tried to pursue this strategy, no publications showing success in larger materials have been presented to date. However, there have been numerous communications stating success in limited series[1] (reviewed by Jackson,[2] Oudejans *et al.*[3] and Hahn *et al.*[4]). The main reason for the lack of success has been the extreme rarity of these fetal cells, which has prevented researchers from isolating a sufficient number of cells to perform a reliable cell typing, which in turn is required for an effective enrichment.

Instead, many groups, with limited success, have tried to establish methods for the enrichment of several different types of fetal cells, of which fetal nucleated red blood cells (erythroblasts) and trophoblasts have been the favorite targets.[2–8] Lymphoid progenitor cells have also been considered in the past; however, in 1996, Bianchi *et al.*[9] demonstrated that these cells can persist in the circulation of parous women years and even decades after pregnancy, making them unsuitable targets for prenatal diagnosis. Finally, studies on fetal cell microchimerism, in both parous mice and women, have shown that the fetal cells that have passed the placenta have a great phenotypic diversity, indicating plasticity of morphology when integrated into maternal tissues, which suggest that fetal cells in maternal blood have stem cell properties.[10–13]

In view of the fact that the fetal cells in maternal blood are still not fully characterized and ideal markers for isolating fetal

cells have not yet been identified, we decided to search for markers specific for fetal cells in maternal blood by going "the long way," namely by isolating a sufficient number of fetal cells from the maternal blood and then isolating messenger RNA (mRNA), synthesizing complementary DNA (cDNA), and performing expression array analysis on this, while using maternal nucleated blood cells as reference in the analysis. We did this by collecting blood samples from a series of pregnant women in gestational week 11–14 with male fetuses, enriching the fetal cells by nonspecific methods or by magnetic-activated cell sorting (MACS) depletion of maternal white blood cells and fixing the remaining cell pellet with fixatives that preserve the cell cytoplasm. Fetal cells were identified by X-chromosome and Y-chromosome FISH followed by automated scanning for Y-chromosome signals. Fetal-derived cells identified in this way were subsequently isolated by laser capture microdissection. mRNA was isolated and used for stem cell marker expression arrays. Similarly, surrounding maternal blood cells were collected as controls.

MATERIALS AND METHODS

Clinical material

The samples used in this study were from 16 women pregnant with a male fetus who came for chorionic villus sampling because of increased risk of carrying a fetus with Down's syndrome. Blood samples, 3–20 ml in heparin-containing tubes for characterization of fetal cells in maternal blood and 5 ml in ethylenediaminetetraacetic-acid-containing tubes for gender determination by PCR, were obtained before chorionic villus sampling.

Informed consent was given by all participants, and the project was approved by the local Danish science ethics committee (Region of Southern Denmark, approval number S-20070045).

Fetal gender determination

Fluorescence in situ hybridization of chorionic villus tissue

A small sample of chorionic villus tissue was smeared onto a microscope slide and allowed to air-dry. The cells were then fixed and permeabilized with methanol. Gender was determined by performing X-chromosome and Y-chromosome FISH as described later.

Polymerase chain reaction procedure

Maternal blood samples were processed 1–4 h after sampling. One milliliter of plasma was used from each sample. Free fetal DNA in plasma was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Subsequently, real-time PCR was performed on 15-μl eluate to detect Y-chromosome-specific genes DYS14 and SRY. HSPA1A was used as the control gene. The amplification was performed on a SmartCycler II (Cepheid, Sunnyvale, CA, USA) in a final volume of 25 μl using OmniMix HS PCR reagent beads (Cepheid) in diethylpyrocarbonate water. Primers, probes, and PCR protocol are given in Table 1.

Preparation of slides for characterization of the fetal cells in maternal blood

For enrichment, fixation, and smearing of fetal cells onto slides, several methods were used.

Sedimentation of blood followed by NH_4Cl-mediated erythrocyte lysis and prefixation

Each blood sample, 3–4 ml for unselected samples and 5–20 ml for samples undergoing MACS, was divided into aliquots of 1 ml and diluted 1:14 with 0.15 M NaCl, and the cells were sedimented over night at 4°C. Twelve milliliters of the supernatant was carefully removed, and 0.5-ml aliquots of sedimented cells were diluted with a 10-ml reagent mixture (10 μM acetazolamide, 13.5 mM NaCl, 0.166 M NH_4Cl). After incubation for 2 min, 200 μl of 30 mM NH_4HCO_3 was added, and the cells were incubated until erythrocytes were lysed (usually 10–15 min). The cells were prefixed in 0.43% formaldehyde for 10 min, centrifuged for 10 min at 500 *g*, washed in 0.15 M NaCl, and recovered by centrifugation for 10 min at 500 *g*.

Table 1 **Probes, primers, and PCR program for gender determination of fetuses**

	Sequence	Final concentration	PCR program
SRY forward primer	5′-TCC TCA AAA GAA ACC GTG CAT-3′	200 nM	One cycle at 95°C for 2 min followed by 45 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s
SRY reverse primer	5′-AGA TTA ATG GTT GCT AAG GAC TGG AT-3′	200 nM	
TaqMan SRY probe	5′-(FAM)-TCC CCA CAA CCT CTT-(TAMRA)-3′	200 nM	
DYS forward primer	5′-GGG CCA ATG TTG TAT CCT TCT C-3′	200 nM	One cycle of 95°C for 1 min followed by 45 cycles at 95°C for 15 s and 60°C for 30 s
DYS reverse primer	5′-GCC CAT CGG TCA CTT ACA CTT C-3′	200 nM	
TaqMan DYS probe	5′-(FAM)-TCT AGT GGA GAG GTG CTC-(TAMRA)-3′	200 nM	
HSPA1A forward primer	5′-AGA AGA CTC TGG AGA GTT C-3′	200 nM	The same as for SRY
HSPA1A reverse primer	5′- TGC CAG GTC GGG AAT ATT CCA-3′	200 nM	
TaqMan HSPA1A probe	5′-(Cal Flour Red 610)-CG TCC TGC CCC CCA GCC TT-(3BHQ)-3′	1 μM	

Positive control: male DNA 5 ng/μl; negative control: DEPC water. Boy fetus if Ct for DYS14 ≤34 and Ct for SRY ≤40; girl fetus if Ct for DYS14 ≥ 36 and Ct for SRY ≥42. PCR, polymerase chain reaction.

For unselected samples, the cell pellet was resuspended in 0.15 M NaCl (approximately 100 μl/ml whole blood) and smeared onto poly-L-lysine-coated microscope slides (10 μl per slide corresponding to approximately 300,000 cells). Slides were air-dried, sealed in airtight plastic bags, and stored at −20°C until hybridization.

For the samples undergoing MACS, the cell pellets (corresponding to 5 ml of whole blood) were resuspended in 80 μl of MACS buffer (phosphate-buffered saline pH 7.2, 0.5% bovine serum albumin, and 2 mM ethylenediaminetetraacetic acid).

Ficoll–Paque separation

Mononuclear cells from 12-ml maternal blood were prepared by using Ficoll–Paque PLUS separation (GE Healthcare, Little Chalfont, UK), according to the protocol provided by the manufacturer. After separation, the cells were prefixed in 0.24% formaldehyde for 10 min and recovered by centrifugation for 10 min at 300 *g*. The cell pellet was resuspended in 80 μl of MACS buffer before magnetic cell separation was performed.

Magnetic-activated cell sorting

Depletion of maternal cells was performed in three different ways using four different kinds of microbeads according to the protocols provided by Miltenyi Biotec GmbH: (1) depletion of CD45-positive cells (product no. 130-045-801) with or without the addition of glycophorin A microbeads (130-050-501), (2) depletion of all white blood cell types except T cells (Pan T, 130-091-156), and (3) depletion of HLA-DR-positive cells (130-046-101). For all three procedures, the cell fraction used for further analysis was the flow-through fraction from LD MACS columns. The resulting cell pellets were resuspended in MACS buffer and smeared on poly-L-lysine-coated microscope slides in 40-μl aliquots (approximately 50,000 cells per slide).

Identification of male fetal cells by XY fluorescence *in situ* hybridization and automated scanning

Slides were fixed, permeabilized, and subjected to FISH as described by Christensen *et al.*[14] Chromosome-specific repeat probes, DXZ1 (CEP X alpha satellite) labeled with spectrum green and DYZ1 (CEP Y satellite III) labeled with spectrum orange (Abbott Molecular, North Chicago, IL, USA), were used for the first hybridization. After FISH, slides were mounted in Vectashield with 0.6 μg/ml 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Cell nuclei containing a red signal were identified by automated scanning at 10× magnification using an in-house developed classifier and the Metafer4–MetaCyte program developed by MetaSystems. The cells selected by the scanners were inspected after automatic relocation on the scanners. Cells that had one red Y signal and one green X signal or a split green X signal were classified as candidate fetal cells.

The true identity of the candidate fetal cells was confirmed by rehybridization with the same probes, labeled with reverse colors using the FISH protocol described earlier (Christensen *et al.*[14] for selective FISH). Rehybridized candidate fetal cells were relocated by the scanner, and FISH signals were inspected. Candidate fetal cells where the red signal had switched to green and the green signal to red were classified as true fetal cells.

Generation of complementary DNA libraries

Laser capture of fetal cells from microscope slides

Twenty-three true fetal cells were relocated and shot off the microscope slides into an adhesive cap of a PCR tube using the Laser Microdissection and Pressure Catapulting technology on a PALM MicroBeam system. All 23 fetal cells were collected in the same cap. For each fetal cell, 100 surrounding maternal cells were collected in the cap of another tube, giving a total of 2300 maternal control cells.

SuperAmp™ RNA amplification

RNA extraction, SuperAmp™ RNA amplification and production, and amplification of cDNA from the RNA were performed on the two samples (23 fetal cells and 2300 maternal cells) by Miltenyi Biotec GmbH (SuperAmp Service). The mRNA was extracted from the cell samples using magnetic beads and transcribed into cDNA using tagged random and oligo(dT) primers. First-strand cDNA was 5′-tagged using eight U terminal deoxynucleotidyl transferases (Fermentas). Tagged cDNA was globally amplified (Expand Long Template PCR System DNA Polymerase Mix, Roche, Basel, Switzerland) using primer complementary to the tag sequence and incubated at 78°C for 30 s, 20 cycles of 94°C for 15 s, 65°C for 30 s, and 68°C for 2 min followed by 21 cycles of 94°C for 15 s, 65°C for 30 s, and 68°C for 2.5 min with an extension of 10 s per cycle and a final step of 68°C for 10 min. PCR product was purified (NucleoSpin® Extract II, Macherey-Nagel, Düren, Germany), and cDNA yield was measured using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the cDNA was checked by running a fraction of the cDNA samples on a gel using the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA).

Complementary DNA microarrays

The PIQOR™ Stem Cell Microarray, containing 930 cDNA oligos relevant for human stem cells and key markers involved in stem cell differentiation, was used in this study. Two hundred fifty nanograms of purified PCR products was labeled either with Cy3-dCTP (maternal cDNA) or with Cy5-dCTP (fetal cDNA) (Amersham, Piscataway, NJ, USA) in a Klenow fragment (10 U) reaction. Labeled cDNAs were purified using the Cy-Scribe GFX Purification Kit (GE Healthcare). Combined and hybridized overnight on 2 PIQOR™ Stem Cell Microarrays using the a-Hyb™ Hybridization Station were 1.25 μg Cy3-labeled and 1.25 μg Cy5-labeled cDNAs. Fluorescence signals of the hybridized PIQOR™ Microarrays were collected using Agilent's Microarray Scanner System (Agilent Technologies). Mean signal and mean local background intensities were obtained for each spot of the microarray image using the

ImaGene® software (BioDiscovery, El Segundo, CA, USA). Low-quality spots were flagged and excluded from data analysis. After background subtraction, unflagged spots were used for calculation of Cy5/Cy3 ratios (mean value of four replicates per gene).

RESULTS

Gender determination of fetuses

In all the samples tested for gender by either FISH of chorionic villus tissue or Y-chromosome quantitative PCR of free fetal DNA isolated from maternal plasma, the outcome of the gender determination was in agreement with the gender found after delivery. For fetal cell enrichment and isolation, we only used blood samples from women carrying a male fetus, so fetal cells could be identified by the presence of XY FISH signals.

Fetal cell enrichment

Nine fetal cells were retrieved from four samples that underwent NH_4Cl-mediated lysis (lysing red blood cells) with no additional enrichment step. However, because of contamination with a huge number of maternal white blood cells, the microscope scannings were very time consuming for these samples. The remaining 12 samples were therefore depleted for a large fraction of maternal white blood cells by MACS before smearing the enriched cells on microscope slides. This reduced the scanning time substantially and resulted in recovery of 14 additional fetal cells. In this depletion procedure, we used markers targeting mainly white blood cells, namely CD45 (resulting in 11 fetal cells), Pan T (resulting in two fetal cells) and HLA-DR (resulting in one fetal cell).

Fetal cell identification

Fetal cell candidates on slides were identified by FISH using probes specific to X and Y chromosomes. Figure 1a and b show a fetal cell candidate among maternal cells after the first FISH and rehybridization FISH, respectively. Cells like this, where both the X signal and the Y signal changed colors after reverse-color FISH, were defined as true fetal cells and used for laser capture microdissection.

Laser capture microdissection and cDNA production

A total of 23 fetal cells were captured by microdissection. The cells that were microdissected were all prepared and initially analyzed 5 to 23 months prior to microdissection. Until microdissection, the slides had been stored at 4°C mounted in Vectashield. After microdissection, RNA extraction, and PCR-based amplification, the amplified cDNA samples were quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies) (Table 2), and the integrity of the cDNA was checked using an Agilent 2100 Bioanalyzer. Figure 2 displays the resulting gel image and electropherograms. The average length of the highly amplified cDNA products ranged between 200 and 1000 bp.

cDNA microarrays

We chose to hybridize the same mixture of labeled fetal and maternal cDNA on two PIQOR™ Stem Cell Microarrays

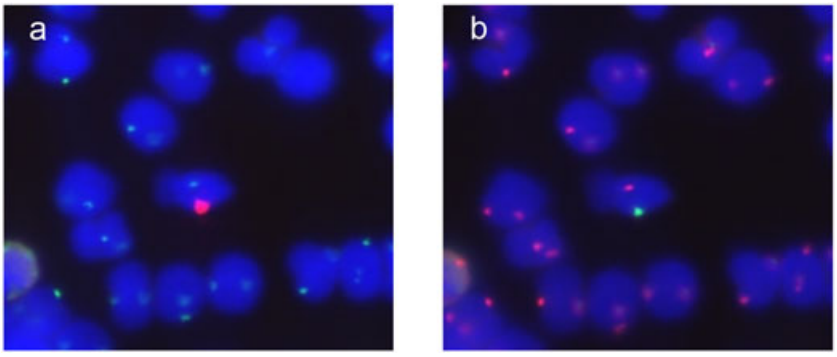

Figure 1 Image of a true positive fetal cell among maternal cells identified by reverse-color FISH using sex-chromosome-specific probes. All cell nuclei were stained blue with nuclear 4′,6-diamidino-2-phenylindole stain. (a) The cells have been subjected to FISH with green probe for the X chromosome (CEP X alpha satellite) and red probe for the Y chromosome (CEP Y satellite III). The fetal cell is the one with a large red Y signal and a small green X signal. (b) The same cells are shown after reverse-color FISH. The Y chromosome in the fetal cell is now green, and the X chromosome in the fetal cell as well as the two X chromosomes in the surrounding maternal cells is now red. The images were captured with the imaging system ISIS (MetaSystems) using a ×40 objective

Table 2 Summary of cDNA yields

Cell sample	Concentration (ng/μl)	Ratio (260/280)	Volume (μl)	Total amount of cDNA (μg)
Maternal control cells (2300)	157	1.8	20	3.1
Fetal cells (23)	161.8	1.72	20	3.2

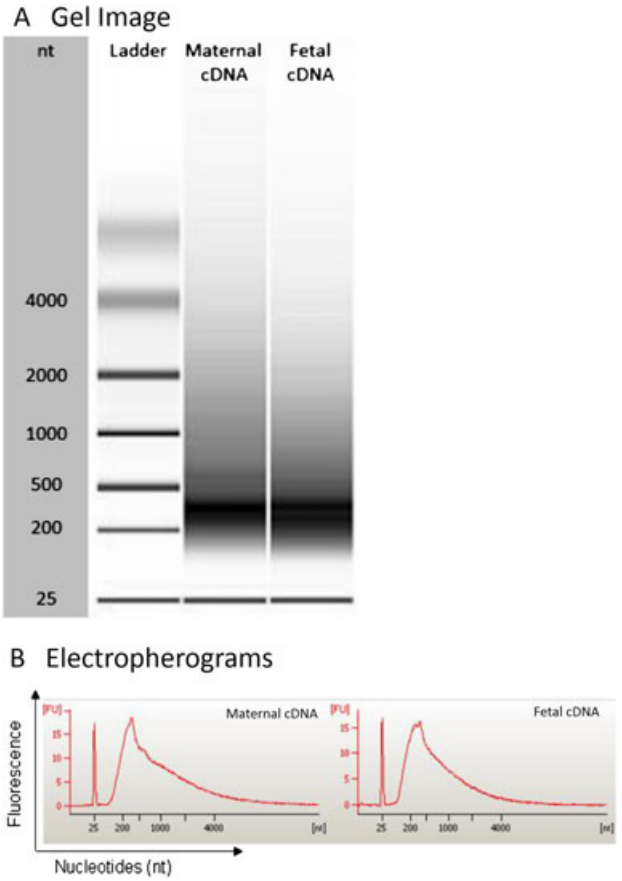

Figure 2 Gel image (A) and electropherogram (B) of amplified cDNA samples. As a reference, the DNA molecular weight ladder (in nucleotides, nt) is shown in the first lane. The lowest migrating band represents an internal standard. The middle lane in (A) and left image in (B) is cDNA made from 2300 maternal cells. The right lane in (A) and right image in (B) is cDNA made from 23 fetal cells. Scaling of the *y*-axis was carried out automatically, relative to the strongest signal within a single run. The image was made by using the Agilent 2100 Bioanalyzer expert software

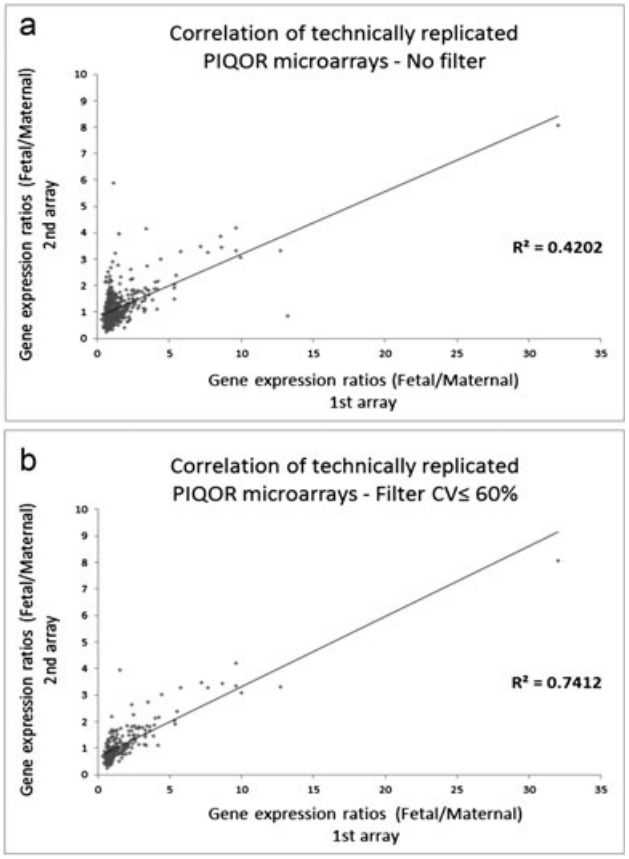

Figure 3 Correlation of the fetal/maternal (Cy5/Cy3) ratios of the two technically replicated PIQOR arrays. Genes that were blank in either array were omitted. (a) Unfiltered ratios are displayed (525 genes are plotted). (b) Gene ratios with no CV or a CV > 60% in either array were omitted (225 genes are plotted). R^2 is the coefficient of correlation for the two datasets

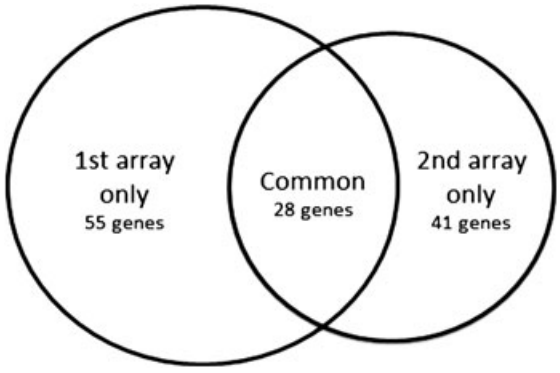

Figure 4 Diagrammatic representation of the two technically replicated microarrays. Number of genes expressed 1.7-fold higher or more in fetal cells compared with maternal cells and filtered to CV ≤ 60%. Fifty-five genes were overexpressed only in the first array, and 41 genes were overexpressed only in the second array. An overlapping group of 28 genes were overexpressed in both arrays

(technical replicates). From the 936 cDNA transcripts printed on the PIQOR™ Stem Cell Microarray, 525 genes were detected in both technically replicated hybridizations. The gene expression ratios (fetal/maternal) of those 525 genes in the two hybridizations are plotted against each other in Figure 3a. The two datasets had a relatively poor correlation coefficient (R^2) of 0.42. For a stricter quality control on the dataset, the result was filtered so that only genes that had a calculated coefficient of variation (CV on four identical spots on the array) lower than 60% were included. Two hundred twenty-five genes detected on both technically replicated microarrays met these criteria, and the correlation coefficient between the two datasets now increased to 0.74 (Figure 3b).

Genes that had more than a 1.7-fold higher expression in fetal cells compared with maternal cells and who had a calculated CV value below 60% were defined as overexpressed genes. In the two array experiments, 83 genes and 69 genes fulfilled these criteria. As shown in Figure 4, a group of 28 genes were overexpressed on both array hybridizations. The protein products of these 28 genes were considered likely fetal cell markers. The 28 genes are listed in Table 3.

Table 3 List of 28 genes overexpressed in both the technically replicated arrays, filtered to CV ≤ 60%

Gene name	First array ratios/% CV	Second array ratios/% CV
MMP14: (MMP14 or MMP-X1) matrix metalloproteinase 14 precursor	32.02/38	8.07/25
CTNNA2: (CTNNA2 or CAPR) alpha-2 catenin	12.70/49	3.31/11
FIGF: (FIGF or VEGF-D) vascular endothelial growth factor D	9.95/18	3.08/22
CRABP1: (CRABP1 or RBP5) retinoic-acid-binding protein I, cellular (CRABP-I)	9.63/37	4.20/14
PBLD: (MAWBP or PBLD) MAWD binding protein	9.62/4	3.33/19
SMC3: (BAMACAN) structural maintenance of chromosome 3	8.66/22	3.44/18
HAMP: (HAMP or LEAP1 or HEPC) hepcidin precursor	7.66/34	3.27/24
CDC42_1: (CDC42) G25K GTP-binding protein, placental isoform	7.19/60	3.47/17
PF4: (SCYB4 or PF4-PF4V1_HUMAN) platelet factor 4 precursor	5.77/48	3.28/46
RSL24D1: (L30) 60S ribosomal protein L30 isolog	5.50/20	2.39/18
CD33: (CD33) myeloid cell surface antigen CD33 precursor	5.38/7	1.91/26
TCF4: (TCF4 or ITF2 or SEF2) transcription factor 4	5.33/10	2.05/9
F11R: (F11R or JAM1 or JCAM) junctional adhesion molecule 1 precursor	4.43/30	3.01/20
MCAM: (MCAM or MUC18) cell surface glycoprotein MUC18 precursor	4.23/20	2.16/32
JUN: (JUN) transcription factor AP-1	4.00/37	2.14/14
JUNB: (JUNB) transcription factor JUN-B	3.94/26	1.88/20
ATF6B: (CREBL1 or G13) cyclic-AMP-dependent transcription factor ATF-6 beta	3.59/29	1.82/11
DLX5: (DLX5) homeobox protein DLX-5	3.49/59	2.75/39
ATF4: (ATF4) cyclic-AMP-dependent transcription factor ATF-4	3.43/6	1.83/19
KCNQ4: (KCNQ4) potassium voltage-gated channel subfamily KQT member 4	3.09/8	1.77/47
CLDN6: (CLDN6) claudin-6 (SKULLIN 2)	2.85/29	1.80/47
TCF3: (TCF3) transcription factor E2-alpha	2.82/25	1.76/12
F3: (F3 or CF3 or CF-3) tissue factor precursor	2.66/46	1.74/45
GDF1: (GDF1 or GDF-1) embryonic growth/differentiation factor 1 precursor	2.51/38	1.86/49
PUM2: (PUM2 or PUMH2 or KIAA0235) Pumilio homolog 2	2.47/11	2.25/38
FGF23: (FGF23 or HYPF) fibroblast growth factor-23 precursor	2.36/19	1.81/32
NTF3: (NTF3) neurotrophin-3 precursor	2.34/16	2.63/50
WNT5A: (WNT5A or WNT-5A) WNT-5A protein precursor	2.02/28	1.83/39

CV, coefficient of variation.

DISCUSSION

To our knowledge, this is the first study to seek information on the fetal cells in the maternal circulation by analyzing their gene expression profile. Because this was performed on "non-selected" samples for the retrieval of nine of the fetal cells and on leukocyte-depleted samples for 14 of the fetal cells used for microarray analysis, it is likely that we have analyzed a mixture of cell types.

Given that we consistently used color swapping in the X-FISH and Y-FISH procedure, we are of the opinion that all 23 cells that we isolated as fetal are in fact of fetal origin. We did, however, worry that the quality of the RNA obtained from cells on the slides would be poor and consequently that the product of the cDNA amplification also would be of low quality with the resulting cDNAs probably representing the most stable and abundant mRNA species in the fetal (and maternal) cells.

As can be observed in Figure 2, the cDNA species that resulted from the amplification represented mRNAs degraded to a size range of only 200–1000 bp, suggesting substantial degradation of the mRNA. Because of this, we did not expect our results to give us the full picture of stem cell gene expression in circulating fetal cells. Rather, we hoped to identify a group of stable mRNAs, which have survived the harsh treatment and which were susceptible to cDNA amplification. The technical replicates of the array analysis showed an acceptable reproducibility, especially if only genes with a CV below 60% were included ($R^2 = 0.74$; Figure 3b), indicating that at least the amplification products were of reasonable quality.

For the expression array analysis, we chose to focus our search on stem cell markers by using the PIQOR™ Stem Cell Microarray. The reason for this choice was that, in numerous studies on fetal cell microchimerism, strikingly different cell types had been found in different organs of women who had been pregnant years before.[11] This diversity of the fetal cells has led to the suggestion that at least a fraction of the fetal cells that pass the placenta have stem cell characteristics.[10]

When analyzing the PIQOR™ Stem Cell Microarray data, we used three criteria when defining genes preferentially

Table 4 **Tissue expression of the 28 fetal gene candidates and information about the function and subcellular localization of their encoded proteins**

Gene name	Protein function (short description from Entrez and/or UniProtKB/Swiss-Prot)	Subcellular localization (UniProtKB/Swiss-Prot)	Tissue expression (BioGPS)
MMP14	Involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling	Cell membrane	Smooth muscle, cardiac myocytes, and uterus corpus
CTNNA2	May function as a linker between cadherin adhesion receptors and the cytoskeleton to regulate cell–cell adhesion and differentiation in the nervous system; regulates morphological plasticity of synapses and cerebellar and hippocampal lamination during development	Cytoplasm. Cytoplasm, cytoskeleton. Cell junction, adherens junction. Cell membrane; peripheral membrane protein; cytoplasmic side Cell projection, axon.	High expression in brain
FIGF (VEGF-D)	The protein encoded by this gene is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family and is active in angiogenesis, lymphangiogenesis, and endothelial cell growth	Secreted	Expressed in lung and adipocytes
CRABP1	This gene encodes a specific binding protein for a vitamin A family member and is thought to play an important role in retinoic-acid-mediated differentiation and proliferation processes	Cytoplasm	Mainly expressed in the thyroid and the retina
PBLD	No information	Unknown	Kidney, fetal liver, small intestine, colon
SMC3	The nuclear form, known as structural maintenance of chromosomes 3, is a component of the multimeric cohesin complex that holds together sister chromatids during mitosis, enabling proper chromosome segregation. Post-translational modification gives rise to the secreted proteoglycan bamacan, an abundant basement membrane protein	Intracellular, nuclear, or secreted protein (dependent on the cell type)	Expressed in a wide range of tissues including blood
HAMP	Involved in the maintenance of iron homeostasis, and it is necessary for the regulation of iron storage in macrophages and for intestinal iron absorption	Secreted	Almost exclusively expressed in the liver
CDC42	A small GTPase of the Rho subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis, and cell cycle progression	Cell membrane; lipid anchor; cytoplasmic side	Expressed in a wide range of tissues, especially high expression in blood cells
PF4	Its major physiologic role appears to be neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation. As a strong chemoattractant for neutrophils and fibroblasts, PF4 probably has a role in inflammation and wound repair	Secreted	CD34+, CD14+ monocytes, and whole blood
RSL24D1	The protein shares a low level of sequence similarity with human ribosomal protein L24. The function of this gene is currently unknown	Nucleus, nucleolus	Expressed in a wide range of tissues, especially high expression in blood cells
CD33	Putative adhesion molecule of myelomonocytic-derived cells that mediates sialic-acid-dependent binding to cells	Cell membrane	CD33+ myeloid, CD14+ monocytes, and whole blood
TCF4	This gene encodes transcription factor 4, a basic helix–turn–helix transcription factor. Binds to the immunoglobulin enhancer Mu-E5/KE5-motif	Nucleus (probable)	Uterus, brain, CD34+ blood, CD19+ B cells, dendritic cells
F11R	An important regulator of tight junction assembly in epithelia. In addition, the encoded protein can act as follows: (1) a receptor for reovirus; (2) a ligand for the integrin LFA1, involved in leukocyte transmigration; and (3) a platelet receptor	Cell junction, tight junction. Cell membrane.	Expressed in a wide range of tissues; highest expression in placenta and bronchial epithelial cells; also expressed in whole blood

Table 4 **Continued**

Gene name	Protein function (short description from Entrez and/or UniProtKB/Swiss-Prot)	Subcellular localization (UniProtKB/Swiss-Prot)	Tissue expression (BioGPS)
MCAM	Plays a role in cell adhesion and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue	Cell membrane	Expressed in a wide range of tissues including placenta and brain; not expressed in blood
JUN	A transcription factor that recognizes and binds to the enhancer heptamer motif 5′-TGA[CG]TCA-3′	Nucleus	Expressed in a wide range of tissues including blood cells
JUNB	Transcription factor involved in regulating gene activity following the primary growth factor response. Binds to the DNA sequence 5′-TGA[CG]TCA-3′	Nucleus	Expressed in a wide range of tissues including blood cells
ATF6B	Transcriptional factor that acts in the unfolded protein response (UPR) pathway by activating UPR target genes induced during endoplasmic reticulum stress	Endoplasmic reticulum membrane	Not highly expressed in any tissues
DLX5	A member of a homeobox transcription factor gene family similar to the *Drosophila* distal-less gene. The encoded protein may play a role in bone development and fracture healing	Nucleus	Specifically expressed in the placenta
ATF4	Transcriptional activator. Binds the cAMP response element (CRE) (consensus: 5′-GTGACGT[AC][AG]-3′), a sequence present in many viral and cellular promoters	Cytoplasm. Cell membrane. Nucleus.	Expressed in a wide range of tissues including blood cells
KCNQ4	The protein forms a potassium channel that is thought to play a critical role in the regulation of neuronal excitability, particularly in sensory cells of the cochlea	Basal cell membrane; multi-pass membrane protein. Note = situated at the basal membrane of cochlear outer hair cells (by similarity)	Not highly expressed in any tissues; highest expression in thalamus
CLDN6	This gene encodes a component of tight junction strands, which is a member of the claudin family	Cell junction, tight junction. Cell membrane; multi-pass membrane protein	Not highly expressed in any tissues
TCF3	The TCF3 gene encodes two basic helix–loop–helix (bHLH) transcription factors, E12 and E47, through alternative splicing, which are involved in regulation of immunoglobulin gene expression. Plays a major role in determining tissue-specific cell fate during embryogenesis, like muscle or early B-cell differentiation	Nucleus	Expressed in CD71+ early erythrocytes and different subgroups of white blood cells
F3	A coagulation factor III, which is a cell surface glycoprotein. This factor enables cells to initiate the blood coagulation cascades, and it functions as a high-affinity receptor for the coagulation factor VII	Cell membrane	High expression in bronchial epithelial cells and retina; lower expression in various other tissues; not expressed in blood cells
GDF1	A member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily. A regulator of cell growth and differentiation in both embryonic and adult tissues	Secreted	Brain
PUM2	Sequence-specific RNA-binding protein that regulates translation and mRNA stability by binding the 3′-UTR of mRNA targets. Its interactions and tissue specificity suggest that it may be required to support proliferation and self-renewal of stem cells by regulating the translation of key transcripts	Cytoplasm (probable)	Expressed in a wide range of tissues; especially highly expressed in the blood and the brain
FGF23	A member of the fibroblast growth factor (FGF) family. FGF family members possess broad mitogenic and cell survival activities and are involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion	Secreted	Not highly expressed in any tissues; highest expression in superior cervical ganglions
NTF3	A member of the neurotrophin family that controls survival and differentiation of mammalian neurons	Secreted	Specifically expressed in cells of the pineal gland
WNT5A	A ligand for members of the frizzled family of seven transmembrane receptors. Can activate or inhibit canonical Wnt signaling, depending on receptor context. Required during embryogenesis for extension of the primary anterior–posterior axis and for outgrowth of limbs and the genital tubercle	Secreted	Specifically expressed in the placenta and the uterus

The information was found on the Entrez, UniProtKB/Swiss-Prot, and BioGPS portals, which were accessed via GeneCards (http://www.genecards.org/).

expressed in fetal cells. Firstly, we selected genes that came up with similar values in the quadruple determinations (CV ≤ 60%), secondly, genes that came up in both technical replicates of the array analysis, and thirdly, genes with a clear difference in expression between fetal and maternal cells (ratio ≥ 1.7). Using these filters, we obtained a set of 28 candidate genes.

From these 28 gene candidates, we wanted primarily to identify genes that coded for proteins that could be used as enrichment markers in MACS-based or fluorescence-activated-cell-sorting-based methods. We also wanted to investigate if a more general overview of the 28 genes could give a hint about the type of the fetal cells.

An ideal protein marker for the enrichment of fetal cells from maternal blood samples by MACS or fluorescence-activated cell sorting should be located on the cell surface of the fetal cells and not be present on maternal blood cells. To investigate if any of the 28 genes code for such proteins, we collected information on their tissue expression and subcellular localization using UniProtKB/Swiss-Prot, Entrez, and the BioGPS portal. This information is listed in Table 4. Nine of the 28 proteins are bound to the cell membrane (apart from the protein product of ATF4, which we exclude because of its main function as a transcriptional activator in the nucleus). Of these nine proteins, we can exclude the protein products of CDC42 and CTNNA2, which are located on the cytoplasmic side of the cell membrane. The remaining seven genes are MMP14, CD33, F11R, MCAM, KCNQ4, CLDN6, and F3. Of these, CD33 and F11R are both widely expressed in blood cells and would therefore not be suitable for enrichment of fetal cells in maternal blood. This leaves us with five genes, MMP14, MCAM, KCNQ4, CLDN6, and F3, which all code for proteins that could be used for fetal cell enrichment. However, it should be pointed out that one cannot be sure that a high mRNA level will translate into a high protein level. This would have to be validated at the protein level.

As to the hints about the fetal cell type, it is evident from Table 4 that the 28 genes have very diverse cellular functions. However, it is somewhat striking that two genes in Table 4 (DLX5 and WNT5A) are almost exclusively expressed in the placenta, that the overexpressed CDC42 gene is the placental isoform, and that another gene (MCAM) have general vascular functions and is highly expressed in the placenta. Although there are too few genes in Table 4 to do a proper bioinformatics analysis, these features taken together do suggest a placental origin of at least some of the fetal cells in maternal blood. This hypothesis is also supported by the finding that six genes (CTNNA2, F3, GDF1, NTF3, PUM2, TCF4) are highly expressed in the brain, which might also point towards the placenta because conserved neuronal pathways have been shown to operate in placental cells.[15]

Table 4 also contains genes that are evidently not expressed in the placenta. Examples are PF4 and TCF3, which are both expressed in blood cells. We interpret this as an indication that there are also other fetal cell types in maternal blood. Nevertheless, from our findings in the present communication, we suggest that at least a fraction of the fetal cells in maternal blood is derived from the placenta and may be useful in non-invasive prenatal diagnosis. As for enrichment markers, we suggest that the protein product of the genes MMP14, MCAM, KCNQ4, CLDN6, and F3 may be suitable for fetal cell enrichment. Of these, MCAM might be the most promising candidate because of the fact that this gene is also highly expressed in the placenta.

ACKNOWLEDGEMENTS

The authors would like to thank Helle Kristensen, Maja Kristensen, Hanne Skanderup, and Simon Tabi Arrey for expert technical assistance. We are also very grateful to Renate Burgemeister and her staff at PALM Microlaser Technologies, a subsidiary of the Carl Zeiss MicroImaging GmbH, Bernried, Germany (now located in Munich, Germany) for great technical assistance and help in using their PALM MicroBeam systems for laser capture microdissection.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Fetal cells are present in the maternal circulation, but which cell type is the most abundant is still unknown.
- Optimal markers for enrichment of a sufficient number of fetal cells from the maternal circulation for reliable fetal-cell-based prenatal diagnostics are lacking.

WHAT DOES THIS STUDY ADD?

- In blood from pregnant women, we identified 28 genes, which were expressed in higher amounts in fetal cells in the maternal circulation than in maternal blood cells. They are candidates for being fetal cell markers.
- Of the 28 proteins encoded by the candidate genes, five may have potential as fetal cell enrichment markers.

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. Jackson L. Fetal cells and DNA in maternal blood. Prenat Diagn 2003;23:837–46.
3. Oudejans CB, Tjoa ML, Westerman BA, et al. Circulating trophoblast in maternal blood. Prenat Diagn 2003;23:111–6.
4. Hahn S, Zhong XY, Holzgreve W. Recent progress in non-invasive prenatal diagnosis. Semin Fetal Neonatal Med 2008;13:57–62.
5. 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.
6. Schueler PA, Yamanishi DT, Pearson J, et al. Inconsistency of fetal trophoblast cells in first trimester maternal peripheral blood prevents non-invasive fetal testing using this cell target. Placenta 2001;22:702–15.

7. Cheung MC, Goldberg JD, Kan YW. Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood. Nat Genet 1996;14:264–8.
8. Prieto B, Candenas M, Ladenson JH, et al. Comparison of different CD71 monoclonal antibodies for enrichment of fetal cells from maternal blood. Clin Chem Lab Med 2002;40:126–31.
9. Bianchi DW, Zickwolf GK, Weil GJ, et al. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci U S A 1996;93:705–8.
10. Lee ES, Bou-Gharios G, Seppanen E, et al. Fetal stem cell microchimerism: natural-born healers or killers? Mol Hum Reprod 2010;16:869–78.
11. Khosrotehrani K, Johnson KL, Cha DH, et al. Transfer of fetal cells with multilineage potential to maternal tissue. JAMA 2004;292:75–80.
12. Fujiki Y, Johnson KL, Peter I, et al. Fetal cells in the pregnant mouse are diverse and express a variety of progenitor and differentiated cell markers. Biol Reprod 2009;81:26–32.
13. Sunami R, Komuro M, Yuminamochi T, et al. Fetal cell microchimerism develops through the migration of fetus-derived cells to the maternal organs early after implantation. J Reprod Immunol 2010;84:117–23.
14. 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.
15. Van DM, Van BJ, Poutsma A, et al. The pre-eclampsia gene STOX1 controls a conserved pathway in placenta and brain upregulated in late-onset Alzheimer's disease. J Alzheimers Dis 2010;19:673–9.

