Identification of candidate surface antigens for non-invasive prenatal diagnosis by comparative global gene expression on human fetal mesenchymal stem cells

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ABSTRACT: Transplacental passage of circulating first-trimester fetal mesenchymal stem cells (fMSC) raises the prospect of harvesting fetal cells in maternal blood. Despite high sensitivity in model systems, negative selection and culture strategies yield fMSC only rarely in post-termination maternal blood. The different adhesion molecule profile of fMSC to competitor maternal cell types suggests that improved positive selection strategies may facilitate non-invasive prenatal diagnosis. We aimed to identify surface antigens specific to fMSC and not maternal peripheral blood lymphocytes (PBL), using genome-wide analysis of actively expressed transcripts. Maternal PBL and fMSC cultured from first-trimester blood, liver and bone marrow were assessed for global gene expression by Affymetrix U133Plus2.0 arrays. Data were analysed using Affymetrix GCOS01.2. Transcripts present in all fMSC (n = 9) but absent in all PBL samples (n = 3) were selected for further analysis of cell-surface membrane molecules by RT–PCR and immunocytochemistry. Of 1544 genes expressed in fMSC and not maternal PBL, filtering for cell-surface molecules yielded 159 genes. Of these, 29 had a mean expression ratio of >3.0 (P < 0.001), which represented 18 unique genes, and their positive expression in all fMSC samples was confirmed by RT–PCR. Candidates for non-invasive prenatal diagnosis were chosen for further analysis by immunocytochemistry. Surface expression of OSMR and COL1 proteins on all fMSC, but no maternal PBL was confirmed. Identification of novel surface antigens on circulating human fMSC and not maternal PBL facilitates positive selection strategies for isolating fMSC for non-invasive prenatal diagnosis.

Key words: aneuploidy / fetal stem cells / feto-maternal trafficking / maternal blood / mesenchymal stromal cells

Introduction

There has been much interest over the last 30 years in the development of techniques for non-invasive prenatal diagnosis. Isolation of fetal cells from maternal blood in early pregnancy would allow exact genetic diagnosis and thus obviate the high false-positive rates and risk of fetal loss associated with current prenatal diagnostic approaches. Aneuploidy screening by serum and/or ultrasound markers has imperfect sensitivity and poor specificity (Nicolaides et al., 2005), while the diagnostic invasive procedures of amniocentesis and chorionic villus sampling (CVS) are offered only to those at high risk of genetic abnormality, because of an inherent 0.5–2% risk of miscarriage (Tabor et al., 1986; Smidt-Jensen et al., 1992; Alfrevic et al., 2003).

Transplacental trafficking of fetal cells into the maternal circulation occurs in all human pregnancies, albeit at low frequency (Ariga et al., 2001; Krabchi et al., 2006). Although various fetal cell types have been isolated in maternal blood such as trophoblasts, leukocytes, nucleated erythrocytes, platelets and haemopoietic progenitors (Covone et al., 1984; Zheng et al., 1993; Little et al., 1997), technical challenges have proved a major obstacle to robust and reproducible isolation of rare-event fetal cells. Non invasive isolation of fetal cells from maternal blood represents the holy grail of prenatal diagnosis, but 20 years of research effort has failed to offer any realistic prospect of a reproducible and accurate method suitable for clinical translation (Jackson, 2003). Admittedly trophoblast DNA in maternal blood has begun to be used clinically, but the low fetal compared with maternal...
DNA yield currently restricts utility to relatively rare indications involving paternally inherited DNA sequences (Fan et al., 2008).

To improve current identification strategies, which are hampered by the extremely low frequency (ca. 1 in 10^6–7) of fetal cells in the maternal circulation (Bianchi et al., 1994), the culture and isolation of mesenchymal stem cells (MSC) has been explored. In model mixtures, one fetal MSC (fMSC) among 10^7 maternal nucleated cells could be isolated with a 100% pure yield of fMSC, to date the most efficient in vitro technique for any fetal cell type (O’Donoghue et al., 2003). However, in clinical samples, the frequency of circulating fMSC in maternal blood and thus yield was very low. Such negative selection approaches prior to fMSC culture were only rarely successful (O’Donoghue et al., 2003), whereas positive selection strategies could prove effective if a definitive fetal cell marker could be identified to select cells for expansion into a pure source of fetal cells, yielding abundant genetic material for prenatal diagnosis.

fMSC represent a promising candidate cell for non-invasive prenatal diagnosis. They are readily isolated from first-trimester fetal blood between 7 and 14 weeks gestation, decline progressively in frequency thereafter (Campagnoli et al., 2000; Yu et al., 2004), implicating them in the establishment of first-trimester haemopoiesis. MSC are not found in the blood of healthy adults (Lazarus et al., 1997), while MSC identified in maternal blood in the first trimester of pregnancy have been shown to be fetal in origin (O’Donoghue et al., 2003).

The late first trimester is the optimal time for prenatal diagnosis, once most of the risk of spontaneous miscarriage has passed but before the window for straightforward methods of termination has ended. Unlike other candidate fetal cells for non-invasive prenatal diagnosis, fMSC have a characteristic morphology and immunophenotype not shared with maternal cells, and are readily expandable in vitro (Campagnoli et al., 2001).

There is now considerable insight into fMSC biology. After MSC were described as multipotent cells with the ability to expand in vitro (Pittenger et al., 1999), human fMSC were first reported by Campagnoli et al. (2001) in first-trimester fetal blood, liver and bone marrow (BM). They grow as spindle-shaped fibroblastic cells similar to adult MSC. They are non-haemopoietic, non-endothelial, consistently negative for CD45, CD34, CD14 CD31 and vWF and express a variety of adhesion molecules including CD44, CD106 (VCAM-1) and CD29 (β-integrin). In their undifferentiated state, fMSC stain positive for laminin and vimentin and mesenchymal markers such as CD73 and CD105. They demonstrate marked expansive capacity, and cycle faster than comparable adult BM-derived MSC, having a doubling time of 30 h over 20 passages (50 population doublings) without senescing or expressing a differentiated phenotype (Campagnoli et al., 2001; Gotherstrom et al., 2005; Guillot et al., 2007). Like their adult counterparts, fMSC possess the ability to differentiate into at least three different mesenchymal tissues: fat, bone and cartilage (Campagnoli et al., 2001). More recent work suggests that they are more primitive than adult MSC, having longer telomeres and expressing embryonic pluripotency markers such as Nanog and Oct4 (Gotherstrom et al., 2005; Guillot et al., 2007), differentiating more readily into bone (Guillot et al., 2008; Zhang et al., 2009) and outside conventional lineage boundaries into skeletal muscle (Chan et al., 2006), and oligodendrocytes (Kennedy et al., 2009).

To find a definitive fetal cell marker not otherwise found in maternal blood, we compared the global genome expression in first-trimester human fMSC derived from fetal blood, liver and BM with that in maternal peripheral blood lymphocytes (PBL). This would be an important translational step in designing new diagnostic assays for fMSC as a potential target for non-invasive prenatal diagnosis in maternal blood in the first trimester.

**Methods**

**Ethics**

Blood and tissue collection for research purposes was approved by the Hammersmith and Queen Charlotte’s Research Ethics Committee in compliance with national guidelines regarding the use of fetal tissue for research (Polkinghorne, 1989). All women gave written informed consent to the additional research procedures.

**Isolation, culture and characterization of human fMSC**

To isolate human fMSC, fetal blood, liver and BM (gestational age 9–13 weeks to 14–20 weeks, Table I) were collected. The fetal tissues were donated from women undergoing terminations for socioeconomic indications in accordance with UK law, and none was known to have any chromosomal or structural abnormalities. Fetal blood was obtained by ultrasound-guided cardiac aspiration before clinically indicated termination of pregnancy. Following the procedure, the liver and BM was collected. Fetal MSC were prepared as described previously (Campagnoli et al., 2001).

Briefly, fetal liver was disintegrated by passage through a 100 μm nylon filter and fetal BM was prepared by flushing long bones with 25 G needles. Single cells were suspended in DMEM-high glucose supplemented with 10% fetal calf serum (Sigma-Aldrich, UK), specifically selected for ultrasound-guided cardiac aspiration before clinically indicated termination of pregnancy. Following the procedure, the liver and BM was collected. Fetal MSC were prepared as described previously (Campagnoli et al., 2001).

Fetal bone marrow #1 10
Fetal bone marrow #2 11
Fetal bone marrow #3 12
Fetal liver #1 9
Fetal liver #2 10
Fetal liver #3 11
Fetal blood #1 10
Fetal blood #2 11
Fetal blood #3 12
Maternal PBL #1 12
Maternal PBL #2 13
Maternal PBL #3 14

<table>
<thead>
<tr>
<th>Source</th>
<th>Gestational age (weeks±days)</th>
<th>Passage number (fMSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal PBL #1</td>
<td>12±0</td>
<td>—</td>
</tr>
<tr>
<td>Maternal PBL #2</td>
<td>13±1</td>
<td>—</td>
</tr>
<tr>
<td>Maternal PBL #3</td>
<td>14±2</td>
<td>—</td>
</tr>
<tr>
<td>Fetal blood #1</td>
<td>10±6</td>
<td>3</td>
</tr>
<tr>
<td>Fetal blood #2</td>
<td>10±6</td>
<td>3</td>
</tr>
<tr>
<td>Fetal blood #3</td>
<td>10±6</td>
<td>3</td>
</tr>
<tr>
<td>Fetal liver #1</td>
<td>9±3</td>
<td>3</td>
</tr>
<tr>
<td>Fetal liver #2</td>
<td>11±5</td>
<td>3</td>
</tr>
<tr>
<td>Fetal liver #3</td>
<td>14±0</td>
<td>4</td>
</tr>
<tr>
<td>Fetal bone marrow #1</td>
<td>10±0</td>
<td>3</td>
</tr>
<tr>
<td>Fetal bone marrow #2</td>
<td>10±4</td>
<td>4</td>
</tr>
<tr>
<td>Fetal bone marrow #3</td>
<td>12±4</td>
<td>5</td>
</tr>
</tbody>
</table>
(Invitrogen) and replated at a density of $4 \times 10^7$ cells/cm$^2$. Detached cells were analysed as detailed next, or re-suspended in PBS and the cell pellets were snap frozen in $-80^\circ$C for later gene expression analysis.

Expression of CD14, CD29, CD31, CD34, CD44, CD45, CD49b/d/e (Pharmingen, UK), CD105, CD106 (VCAM-I), vimentin, laminin, vWF, HLA class I, HLA class II (DR) (DAKO, UK), SH3 and SH4 (CD73, Osiris, USA) by fMSC was analysed separately on a fraction of the cultured cells using mouse monoclonal antibodies and osteogenic, chondrogenic and adipogenic differentiation was determined in lineage directed assays as previously described (Campagnoli et al., 2001).

Collection of PBL
Peripheral blood was collected from three pregnant women with continuing pregnancies (gestational weeks 12$^{+0}$, 12$^{+10}$ and 13$^{+1}$, Table I). Thus, these were not from the same pregnancies that sourced the fetal tissues. Adult maternal PBL were prepared by centrifuging peripheral blood layered on a Ficoll gradient (Invitrogen) at 500g for 20 min at room temperature. The interphase was collected, washed twice in phosphate buffer saline (PBS) by centrifugation and then re-suspended in PBS. Mononuclear cells were counted and the cell pellets snap frozen in $-80^\circ$C for later gene expression analysis as described below.

Whole-genome expression array analysis
After thawing, total RNA from all samples was extracted using the Qiagen RNeasy kit (Qiagen, UK) according to manufacturer’s instructions. Isolated RNA was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) to verify sample integrity and quantify total RNA yield. Ten micrograms of total RNA were converted to cDNA and following second-strand cDNA synthesis (GeneChip One-cycle cDNA synthesis kit, Affymetrix, Santa Clara, Calif, USA) and clean-up (GeneChip Sample Cleanup Module, Affymetrix), biotin-labelled anti-sense cRNA were generated by in vitro transcription with a commercially available kit (GeneChip IVT Labelling Kit, Affymetrix). After clean-up of the labelled cRNA (GeneChip Sample Cleanup Module, Affymetrix), samples were again analysed using the Agilent 2100 Bioanalyzer to verify the quality of the cRNA. Next, 45 µg of each generated cRNA preparation was fragmented and hybridized to HU-U133Plus2.0 GeneChips (Affymetrix), which contain over 55 000 probe sets representing more than 47 000 transcripts derived from 38 500 well-characterized human genes. After automated washing, arrays were stained with streptavidin–phycoerythrin (Molecular Probes) and scanned with a confocal laser GeneChip scanner (Affymetrix). GeneChip Operating Software 1.2 (GCOS, Affymetrix) was used to derive signal intensities.

Data were analysed as follows: (i) the mean expression ratio and P-value were calculated for each transcript, (ii) all transcripts that scored as present in all fMSC and absent in all maternal PBL were selected, (iii) these transcripts were then filtered for molecules involved in cell-surface-linked signal transduction and antigen presentation and (iv) transcripts with a mean expression ratio of $>300$ were further analysed.

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE18934 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18934).

Reverse transcriptase–polymerase chain reaction
To confirm array findings, we performed reverse transcriptase–polymerase chain reaction (RT–PCR) on samples extracted at the same time as those used in the array analysis. Total RNA from the cells was harvested by using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. cDNA was obtained by reverse transcription using 2 µg of total RNA with a kit (A3500, Promega, UK) according to manufacturer’s instructions. PCR reactions were carried out with 1 µl cDNA using specific primers (Table II). The PCR reactions began with an initial 2 min denaturing step and included 10 cycles (94°C for 10 s and 63°C for 60 s) and 25 further cycles (94°C for 10 s, 59°C for 50 s and 72°C for 30 s). Negative control was dH2O instead of cDNA. Amplification of cDNA without reverse transcription or using genomic DNA did not generate any products in PCR reactions (data not shown). The house keeping gene c-abl oncogene 1, receptor tyrosine kinase (ABL1) was used as a loading control.

Immunocytochemistry
To confirm both the expression array and RT–PCR findings, we analysed the surface expression of six proteins on fMSC and maternal PBL. Fetal blood, liver and BM MSC were cultured in 8-well chamber slides (Lab-Tek II RS glass slide, Nunc, Roskilde, Denmark) as described above. All following steps were carried out at room temperature, unless otherwise stated. At sub-confluence, fMSC were washed three times in PBS and fixed in ice-cold methanol for 5 min. PBL from the pregnant women were cytospun onto glass slides, air-dried and fixed as fMSC. The cells were washed in PBS and endogenous peroxidases blocked with 0.3% H2O2 for 3 min, washed again in PBS before blocking for 1 h in the same serum as the primary antibody was diluted in. Primary antibodies were anti-human COL1, FN (both 1:100 in 5% horse serum) (Sigma-Aldrich), WNT5A, OSMR (both 1:50 in 5% goat serum), DKK1 (1:50 in 5% rabbit serum) (Santa Cruz Biotechnology, Santa Cruz, USA), PDGFRA (1:100 in 5% rabbit serum) (Abcam, Cambridge, UK) and were applied overnight at 4°C. Cells were washed in PBS three times and either a horse anti-mouse, horse anti-goat or goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, UK) diluted at 1:100 in 5% indicated serum in PBS was applied for 30 min. The cells were then further washed three times in PBS followed by the avidin:biotinylated enzyme complex (ABC standard Elite kit, Vector) for 30 min, washed three times with PBS and subsequently labelled with DAB substrate (Vector) until desired colour appeared. Cells were then washed in H2O and mounted with 100% glycerol and analysed by light microscopy. Incubation with secondary antibody only served as a negative control.

Results
Fetal MSC
Human fMSC from first-trimester blood, liver and BM were successfully isolated and expanded. Before passage 1, the cells grew in well-defined colonies, which consisted of a few dozen to several hundred cells. They did not express haematopoietic markers (negative for CD14, CD45, CD31 and vWF), and were uniformly positive for the mesenchymal cell type markers CD29, CD44, CD106 (VCAM-I), CD105, SH3 and SH4 (CD73), including CD49b/d/e, laminin and vimentin. Fetal MSC expressed low levels of HLA class I and no HLA class II on the cell surface as previously reported (Campagnoli et al., 2001; Gotherstrom et al., 2004). Staining of extracellular calcium with the von Kossa method or with Alizarin Red S showed osteogenic differentiation in induced cultures. When plated on adipogenic media, fMSC produced lipid-containing vacuoles that could be detected with Oil Red O staining. Chondrogenic differentiation in micromass pellet cultures stained positive for Collagen type 2 and Safranin O as previously reported (Campagnoli et al., 2001).
Whole-genome expression array analysis

A dendrogram, which displays true multi-object data, was created using Euclidean distances (Fig. 1). This branching tree-like diagram illustrates the hierarchical relationships among items in a data set, with the distance from the root to a cluster, indicating the similarity of the clusters. Highly similar clusters have joining points that are farther from the root. The branches represent clusters obtained on each step of hierarchical clustering. The dendrogram in Fig. 1 corroborated an overall
difference in gene expression between fMSC from all sources with maternal PBL since they clearly cluster in two separate groups.

Of 1544 transcripts expressed in fMSC and not maternal PBL, filtering for cell-surface molecules yielded 159 transcripts. Of these, 29 had a mean expression ratio of $>300$ ($P < 0.001$) in fMSC compared with maternal PBL. Of these 29 transcripts, 18 were unique genes, as some genes were represented several times among the top 29 transcripts. These most abundantly expressed cell-surface genes in fMSC compared with maternal PBL are shown in Table III.

**Confirmation of genome expression array results**

The 18 unique genes with a mean expression ratio of $>300$ in the array analysis were validated with RT–PCR which confirmed expression of all 18 genes in all three fMSC populations (Fig. 2). However, of these, only nine (CPE, CRYAB, DKK1, FG5, GNG12, ADAMTS1, PDGFRA, SLIT2 and WNT5A) were negative in all maternal PBL. Nine genes (COL1, FN, FG5, STC2, CAP2, LOX, CXCL12, INHBA and ADAMTS1) were positive in all maternal PBL compared with fMSC.

Of the 18 genes in the array analysis, confirmation of protein expression of six proteins (COL1, FN, WNT5A, DKK1, OSMR, PDGFRA) was performed by immunocytochemistry, where antibodies were commercially available. All sources of fMSC strongly expressed COL1, OSMR (Fig. 3), FN, WNT5A and DKK1 (not shown). PDGFRA were expressed at low levels (not shown). Maternal PBL did not express COL1, OSMR (Fig. 3) or DKK1, all were positive for WNT5A and PDGFRA and a small population expressed FN (not shown).

### Discussion

In order to overcome the main barriers to progress with cell-based non-invasive prenatal diagnosis, we investigated whether specific surface markers can be identified on fMSC. MSC can be found in the circulation in significant amounts during the first trimester of pregnancy, but are otherwise not detectable in adult peripheral blood (Lazarus et al., 1997; Wexler et al., 2003). In addition, they have large self-renewal capacity, rendering them promising target cells for non-invasive prenatal diagnosis. With existing negative selection strategies, fMSC could only be identified in maternal blood occasionally (ca. 5%), even after the 80-fold increase in feto-maternal haemorrhage that occurs post-surgical termination of pregnancy, under which the selection strategy was tested (O’Donoghue et al., 2003). Alternative enrichment strategies, such as positive selection for fMSC cell-surface antigens, might allow their isolation in greater numbers from vastly more abundant maternal cell populations.

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**Table III Overview of cell-surface transcripts expressed in fMSC but not maternal PBL.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>P-value</th>
<th>Mean expression ratio</th>
<th>Affymetrix probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>COL1</td>
<td>Collagen type I alpha 2</td>
<td>&lt;0.00001</td>
<td>5334.9; 1235.0</td>
<td>202404_s_at; 202403_s_at</td>
</tr>
<tr>
<td>2</td>
<td>FN</td>
<td>Fibronectin I</td>
<td>&lt;0.00001</td>
<td>4274.1; 3453.6; 2029.4; 1062.5; 374.6</td>
<td>211719_x_at; 210495_x_at; 212464_s_at; 216442_x_at; 214701_s_at</td>
</tr>
<tr>
<td>3</td>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)</td>
<td>&lt;0.00001</td>
<td>2494.6</td>
<td>209687_at</td>
</tr>
<tr>
<td>4</td>
<td>INHBA</td>
<td>Inhibin beta A (activin A activin AB alpha polypeptide)</td>
<td>&lt;0.00001</td>
<td>1870.8; 889.5</td>
<td>227140_at; 210511_s_at</td>
</tr>
<tr>
<td>5</td>
<td>RGS4</td>
<td>Regulator of G-protein signalling 4</td>
<td>&lt;0.00001</td>
<td>1778.9; 911.9</td>
<td>204337_at; 204338_s_at</td>
</tr>
<tr>
<td>6</td>
<td>STC2</td>
<td>Stanniocalcin 2</td>
<td>&lt;0.00001</td>
<td>1181.3</td>
<td>203438_at</td>
</tr>
<tr>
<td>7</td>
<td>DKK1</td>
<td>Dickkopf homolog 1</td>
<td>&lt;0.00001</td>
<td>885.5</td>
<td>204602_at</td>
</tr>
<tr>
<td>8</td>
<td>WNT5A</td>
<td>Wingless-type MMTV integration site family member 5A</td>
<td>&lt;0.00001</td>
<td>878.1; 307.3</td>
<td>213425_s_at; 205990_s_at</td>
</tr>
<tr>
<td>9</td>
<td>LOX</td>
<td>Lysyl oxidase</td>
<td>&lt;0.00001</td>
<td>702.1; 495.1; 456.2</td>
<td>204298_s_at; 213640_s_at; 215446_s_at</td>
</tr>
<tr>
<td>10</td>
<td>CRYAB</td>
<td>Crystallin alpha B</td>
<td>&lt;0.00001</td>
<td>580.4</td>
<td>209283_at</td>
</tr>
<tr>
<td>11</td>
<td>FG5</td>
<td>Fibroblast growth factor 5</td>
<td>&lt;0.00001</td>
<td>444.8; 346.1</td>
<td>210310_s_at; 210311_at</td>
</tr>
<tr>
<td>12</td>
<td>GNG12</td>
<td>Guanine nucleotide binding protein (G protein) gamma 12</td>
<td>0.0002</td>
<td>423.4</td>
<td>212294_at</td>
</tr>
<tr>
<td>13</td>
<td>ADAMTS1</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif 1</td>
<td>&lt;0.00001</td>
<td>374.7</td>
<td>222486_s_at</td>
</tr>
<tr>
<td>14</td>
<td>PDGFRA</td>
<td>Platelet-derived growth factor receptor alpha polypeptide</td>
<td>0.00003</td>
<td>367.5</td>
<td>203131_at</td>
</tr>
<tr>
<td>15</td>
<td>CPE</td>
<td>Carboxypeptidase E</td>
<td>&lt;0.00001</td>
<td>347.6</td>
<td>201116_s_at</td>
</tr>
<tr>
<td>16</td>
<td>SLIT2</td>
<td>Slit homolog 2</td>
<td>&lt;0.00001</td>
<td>316.7</td>
<td>209897_s_at</td>
</tr>
<tr>
<td>17</td>
<td>OSMR</td>
<td>Oncostatin M receptor</td>
<td>&lt;0.00001</td>
<td>312.4</td>
<td>226621_at</td>
</tr>
<tr>
<td>18</td>
<td>CAP2</td>
<td>CAP adenylate cyclase-associated protein 2</td>
<td>&lt;0.00001</td>
<td>300.3</td>
<td>212554_at</td>
</tr>
</tbody>
</table>
Using a genome-wide expression array technique, we identified differentially expressed genes associated with surface membrane proteins up-regulated in fMSC and not maternal PBL, and confirmed this through RT–PCR and immunocytochemistry. PBL in the maternal blood were positive for the proteins PDGFRA and FN on immunostaining, which detracts from these molecules’ potential as selection targets for non-invasive prenatal diagnosis. WNT5A and DKK1 are both secreted proteins, rendering them not useful for this purpose. However, OSMR and COL1 were both positive by immunostaining in all fetal samples, and were not expressed on maternal PBL. Given the already well-developed antibodies to these cell-surface markers, they appear the best antigens for new approaches to isolate fMSC from maternal blood.

There are several limitations to this study. With microarrays, the gene expression profiles obtained may not entirely correlate with RT–PCR or protein expression validation. A recent review suggests that composed microarray data reliably demonstrate the existence and direction of transcriptional changes for relatively abundantly expressed transcripts, whereas consistent results are more challenging for genes expressed at low levels (Draghici et al., 2006). This is true for the present study; all data on fMSC where genes were abundantly expressed corresponded, both in the microarray and in the RT–PCR and protein analysis, whereas half of the transcripts that scored as absent in the PBL in the microarray showed expression in RT–PCR.

Another possible limitation is that fMSC could be present in the sampled maternal blood. Although the frequency of fMSC in uncultured maternal PBL samples should be exceedingly low, especially since these pregnancies were ongoing, with no feto-maternal haemorrhage, any fetal cell should not alter the gene expression profile in cRNA sourced from thousands of maternal cells, especially since we

**Figure 2** Results from whole-genome expression analysis are validated by RT–PCR.

RT–PCR analysis of fMSC from blood (BL# 1, 2, 3) and liver (Li# 1, 2, 3), bone marrow (BM# 1, 2, 3) and maternal PBL (PBL# 1, 2, 3). fMSC express all genes, whereas maternal PBL do not. The negative control (Neg C) shows RT–PCR using dH2O. The housekeeping gene ABL was used as a loading control. fMSC, fetal mesenchymal stem cells; PBL, peripheral blood lymphocytes.
only searched for abundantly expressed markers. We deliberately
studied pregnant maternal blood as the appropriate comparator, as
pregnancy induces considerable and sometimes profound changes in
both gene expression and circulating cell types.

We acknowledge that fMSC have recently been isolated from first-
trimester placenta (Portmann-Lanz et al., 2010), raising the possibility
that these cells might also be present in maternal blood. However,
fMSC have only ever been identified in maternal blood in the first tri-

term when fetal blood is rich in fMSC (Campagnoli et al., 2001, O’Donoghue et al., 2003), whereas placental MSC can be isolated
from the placenta throughout gestation. In addition, several reports
of alleged placental MSC have turned out to be maternal in origin
(In’t Anker et al., 2004; Barlow et al., 2008; Brooke et al., 2009; Semenov et al., 2010). Moreover, the trafficking of placental cells
into maternal blood has been demonstrated for the overlying epithelial
trophoblast, but not core mesenchymal (such as MSC) or endothelial
components. Nevertheless, we acknowledge this possibility and that
placental MSC might have a different gene expression to fMSC,
although data from this study suggest a consistent gene expression
in fetal MSC independent of tissue source.

The potential of OSMR and COL1 as candidates for fMSC enrich-
ment protocols now warrants evaluation in both spiking experiments
with adult nucleated cells, and then isolation from first-trimester post-
termination maternal blood, before considering testing in phase I clini-
cal studies. Because of the considerable technical challenge in very rare
event isolation, we report our findings at this stage. We acknowledge
the possibility that fetal MSC, which are only present in the maternal
circulation in extremely low numbers, might still be below the level of
sensitivity of the selection method. A further remote possibility is that
fMSC from a previous pregnancy might persist in an undifferentiated
state in maternal blood, as they can be found in tissues (but not blood)
of post-reproductive women (O’Donoghue et al., 2004; Santos et al., 2008).

At present, the rarity of fMSC in maternal blood and the limitations
of negative selection approaches have hampered their clinical trans-
lation in non-invasive prenatal testing. The differential cell-surface
markers determined in this study may allow new positive selection
strategies to be developed for the isolation of fMSC in maternal blood.

**Authors’ role**

C.G., J.C. and K.O. designed, performed and analysed the exper-
iments, wrote and revised the manuscript. N.M.F. designed exper-
iments, wrote and revised the manuscript.

**Conflict of interest:** N.M.F. was a Director of RevealCyte Ltd from
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Non-invasive prenatal diagnosis using fetal MSC


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