Isolation and analysis of intact fetal cells in maternal blood remains an attractive but unrealized approach for performing non-invasive genetic testing. Intact fetal cells could be analyzed either directly or used as a complementary or additive assay with ultrasound or maternal serum analyte
suitable for subsequent FISH analysis. Maternal cells using CD45, and yields cells highly different than those enriched after depletion of necessarily of the erythroid lineage, are clearly neither MACS, targets progenitor cells that are not depletion method is rapid, requires neither MACS from whole blood specimens. This density-based enrichment assay for recovery of intact fetal cells has yet to be developed. Major difficulties in developing an optimal enrichment approach are fetal cell loss during enrichment, overall rarity of fetal cells and lack of a fetal cell-specific marker.

Many different enrichment methods have been used to reduce the overall number of maternal cells while maintaining maximum yield of fetal cells. The two most commonly described approaches involve magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS). Both typically involve sequential steps, such as preliminary separation to remove mature red blood cells (RBC) and granulocytes followed by antibody labeling to achieve negative and/or positive selection to remove unwanted maternal cells. Unfortunately, each step of the MACS- and FACS-based enrichment procedures has the potential of reducing fetal cell recovery. Rarity of fetal cells in maternal blood has complicated the development of an optimal method. One commonly cited estimate is that 1–2 fetal cells exist per 1 cc of maternal blood (1), yet numerous studies indicate recovery of only 1–3 fetal cells per maternal blood specimen (10–30 cc). Given that previous data were based on calculations of DNA equivalents using quantitative PCR, it is plausible that even the 1–3 cells per 30 cc overestimates the number of actual intact fetal cells. Indeed, most fetal DNA could actually be circulating cell-free fetal DNA (2).

Choice of target cell type further influences sensitivity of fetal cell detection. Most investigators have focused on enrichment of the fetal nucleated red blood cells (nRBC) (3–9). Despite attractiveness for selection, fetal nRBC may not be ideal. Only a portion of maternal blood nRBC is fetal (10), and not all are suitable for interphase FISH analysis (11–12). An alternate fetal cell type may be more suitable for recovery and analysis. For these reasons, we developed a new simplified approach using the RosetteSep™ progenitor enrichment assay for recovery of intact fetal cells from whole blood specimens. This density-based depletion method is rapid, requires neither MACS nor FACS, targets progenitor cells that are not necessarily of the erythroid lineage, are clearly different than those enriched after depletion of maternal cells using CD45, and yields cells highly suitable for subsequent FISH analysis.

Materials and methods

Blood samples

After institutional review board approval and written informed consent, 20–30 ml of peripheral blood was collected in acid citrate (solution A; ACD) vacutainers from a total of 81 pregnant women prior to an invasive prenatal genetic diagnostic procedure (34 chorionic villus samples; 47 amniocentesis) Mean gestational age was 15.4 weeks ranging from 10.1 to 23.1 weeks. All specimens were coded and delivered to the laboratory for blinded preparation and analysis. Cord blood specimens (n = 7) were also collected in ACD vacutainers after normal term delivery.

Non-MACS-based enrichment

Maternal blood specimens were randomly assigned within 4–24 h to one of two density-based enrichment methods: (1) Progenitor cell enrichment: samples (n = 67; 15.4 mean gestational weeks, range 10.1–23.1) were labeled with a RosetteSep™ progenitor antibody cocktail (StemCell Technologies; Vancouver, Canada) specific to CD2, CD3, CD14, CD16, CD19, CD24, CD56 and CD66b. The goal was to remove unwanted mature T-cells, B-cells, granulocytes, natural killer cells, neutrophils and myelomonocytic cells. (2) CD45 negative cell enrichment: samples (n = 14; 15.4 mean gestational weeks, range 10.7–20.4) were labeled with the RosetteSep™ CD45 antibody cocktail (Stem-Cell Technologies) specific to CD45 and CD66b to remove unwanted maternal leukocytes of all lineages. The RosetteSep™ CD45 antibody therefore removed many more maternal cells than did the progenitor antibody cocktail.

To minimize potential contamination of recovered cells with cell-free DNA, plasma was removed from whole blood samples after a 10-min centrifugation at 1100 g. Fifty microliters of antibody cocktail per milliliter of blood (original volume) was added to the remaining blood and incubated for 20 min at room temperature. This permitted formation of rosettes by cross-linking unwanted cells to RBC, thereby effectively increasing cell density. The labeled blood was then diluted in phosphate-buffered saline containing 2% fetal bovine serum (PBS/FBS), layered over a Ficoll (1.077 g/ml; Stemcell Technologies, Inc., Vancouver, B.C.) gradient and subjected to a single centrifugation at 1200 g for 20 min. Cells in rosette configuration pelleted with red blood cells, leaving a banded cellular layer above the pellet. The desired non-labeled and less dense cellular fraction was recovered, washed once with PBS/FBS and then placed either on slides for FISH (n = 63; 49 from progenitor and 14 from CD45-negative enrichment) or in microfuge tubes for DNA extraction and real-time PCR (n = 18; all from progenitor enrichment).
Fluorescence in situ hybridization

Recovered cells were fixed onto slides after a 15-min incubation in fresh Carnoy’s fixative (1:3 methanol/glacial acetic acid). Slides were allowed to air dry and stored at −20°C for batch processing. Prior to FISH, slides were removed from −20°C and brought to room temperature. Slides were dehydrated after sequential 70%, 90% and 100% ethanol incubations, and finally air-dried.

For a 10-µl reaction cocktail, direct-labeled probes (Vysis, Inc., Downers Grove, IL) were combined using 1.0 µl of chromosome 21 (Spectrum Orange), 0.3 µl of chromosome X (1:1 Spectrum Orange: Spectrum Green) and 0.3 µl of chromosome Y (Spectrum Aqua). The cocktail was denatured separately in a 68°C water bath for 4 min then applied to a 37°C pre-warmed coverslip. Coverslips and probes were sealed onto slides using rubber cement and subjected to simultaneous denaturation at 80°C for 1 min. Following an overnight incubation in a 37°C humidified chamber, coverslips were removed and slides washed in 0.4× SSC at 65°C for 30 s. DAPI (4′,6-diamidino-2-phenylindole II; Vysis, Inc.) counter stain was applied and cells viewed under a Axiophot fluorescent microscope (Carl Zeiss, Thornwood, NY) equipped with triple band pass filters that allowed simultaneous visualization of red, yellow, green and blue fluorescent signals. For each sample, a maximum of 3000 nuclei were scored, representing from 25% to 50% of the total number of cells recovered.

DNA extraction and real-time PCR

Of the 67 progenitor enriched cases, 18 were randomly selected and subjected solely to real-time PCR analysis. The entire cellular fraction underwent cellular lysis and DNA extraction using the QIAamp Blood Kit (Qiagen, Basel, Switzerland). Real-time PCR using the TaqMan Assay (Applied Biosystems, Foster City, CA) was performed using the GeneAmp 7700 Sequence Detection System (Applied Biosystems). Quantification of both the Y chromosome-specific DYS1 (Y49A clone) and the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences was performed as surrogates for fetal and maternal DNA, respectively (1, 2). Sufficient DNA from each sample was aliquoted to allow up to 30 amplifications. For the GAPDH control locus, 9 amplifications of each sample were performed. For DYS1, analysis was based on 12–21 amplifications, representing from one- to two-thirds of the total DNA sample. A sample was classified as male when >2 amplifications were positive for DYS1 sequences. To control for DNA contamination, DNA extractions and PCR were performed under sterile conditions and by a female technician. In addition, multiple DYS1 negative female DNA and water blank controls were used throughout the study.

Flow cytometric analysis

Split cord blood samples (n = 7) were processed using both the RosetteSep™ progenitor and the CD45-negative enrichment method. To delineate the types of cells recovered by both methods, cells were stained in triple color combinations and analyzed by flow cytometry. Cells were stained using monoclonal antibodies specific CD14.FITC, CD13.PE, CD45.ECD, CD34.FITC, CD33.PE (Beckman Coulter, Miami, FL), as well as CD34.PE (BD Pharmingen, San Diego, CA). These markers identify subsets of progenitor and myeloid lineage specific cells. Table 1 summarizes the antibody phenotype with corresponding cell lineage. CD34 is common to all primitive progenitor cells. Although CD45 is specific predominantly to white blood cells, other cells (i.e. myeloid cells) may also weakly express this antigen. CD14 is common to monocytes and tissue macrophages. CD13 antibody is a common marker of committed myeloid cells.

Results

Subsequent to analysis, results from cytogenetic analysis of chorionic villus or amniotic fluid cells were obtained. The maternal samples included 71 euploid singleton cases (42 female, 29 male), 4 twins (one same sex 46,XX and 46,XX; two unlike sets 46,XX and 46,XY; one set of 47,XY,+13 and 46,XY), one triplet (46,XX/46,XY/unknown) and five karyotypically

Table 1. Phenotypic markers on myeloid lineage cells

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lineage characteristics</th>
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<tbody>
<tr>
<td>CD34 CD45</td>
<td>White blood cell progenitors</td>
</tr>
<tr>
<td>CD33 CD34</td>
<td>Myeloid progenitors, monocytes, macrophages</td>
</tr>
<tr>
<td>CD13 CD14</td>
<td>CD13-committed myeloid lineage marker</td>
</tr>
<tr>
<td></td>
<td>CD14 monocytes and tissue macrophages</td>
</tr>
</tbody>
</table>

Improved isolation of fetal cells from maternal blood

CD33 CD45 White blood cell progenitors
CD33 CD34 Myeloid progenitors, monocytes, macrophages
CD13 CD14 CD13-committed myeloid lineage marker
CD14 monocytes and tissue macrophages
abnormal cases (45,X; 46,XX,t[5;18]; 47,XY,þ21; 45,X/46,XX; 46,XY/47,XY,þ21).

Eighty percent correct detection of fetal gender was observed with the RosetteSep™ progenitor cell enrichment approach (39 out of 49 cases, Table 2). In 10 out of 19 (53%) pregnancies confirmed to have a male (XY) fetus, an average of 2–3 intact fetal cells containing one signal for the X and Y chromosomes was detected. False-positive detection of male (XY) cells occurred in 1 out of 30 (3%) female cases. Two and three fetal male (XY) cells were detected in one normal (46,XX and 46,XY) and one abnormal (46,XY and 47,XY,þ13) twin pregnancies, respectively. In contrast, no fetal XY cells were detected in seven male cases processed with CD45-negative RosetteSep™ enrichment. Fetal cells were also not detected in either the triplet pregnancy or the 47,XY,þ21 case using the CD45-enrichment approach.

The majority of cells recovered after RosetteSep™ enrichment was of good to excellent quality based on criteria of an intact nucleus and clarity of nuclear stain using DAPI. This contrasts with our experience using FACS and parafomaldehyde fixation (13). Hybridization efficiency was 99% for X and Y probes compared with 93% for the locus-specific 21 probe. With progenitor cell enrichment, 6000–12,000 (mean 6200) cells were recovered from each case, which began with 20–30 ml of maternal blood. Fewer cells were recovered with the CD45 enrichment, ranging from 5146 to 150 cells (mean 2847). That no fetal cells were detected by the CD45-negative enrichment method suggests that the target fetal cells were lost during enrichment.

To determine whether fetal male cells could be present but go undetected by FISH with the RosetteSep™ progenitor enrichment approach, real-time PCR was performed on all cells recovered from 18 prospective maternal cases. Among the nine cases confirmed to have a male fetus, eight (89%) cases were correctly identified based on positive DYS1 detection in >2 amplifications wells (Table 3). No false-positive detection was observed. Among the male cases, mean fetal sequences detected were in the range of 0.067–1.167 genome equivalents per milliliter of blood (Geq/ml). Given that 12–21 amplifications per sample were performed for the DYS1 locus, greater than one- to two-thirds of the DNA sample was tested for fetal sequences. Based on these results, a mean of 9.1 (range 2–35) fetal progenitor cells are estimated to be present in a 30-ml maternal blood sample.

<table>
<thead>
<tr>
<th>Fetal gender</th>
<th>FISH on RosetteSep™-enriched cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male cases (n = 26)</td>
<td>10/19 (53%)</td>
</tr>
<tr>
<td>Female cases (n = 37)</td>
<td>29/30 (97%)</td>
</tr>
<tr>
<td>Total (n = 63)</td>
<td>39/49 (80%)</td>
</tr>
</tbody>
</table>

Table 3. Real-time PCR detection of fetal DNA sequences (DSY1) in intact cells recovered from confirmed male maternal blood cases after RosetteSep™ progenitor cell enrichment

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Mean a (Geq/ml)</th>
<th>Range (Geq/ml)</th>
<th>Expected no. fetal cells b</th>
<th>Mean (Geq/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10246</td>
<td>0.267</td>
<td>0.1–0.5</td>
<td>8</td>
<td>6613</td>
</tr>
<tr>
<td>10247</td>
<td>1.167</td>
<td>0.3–2.4</td>
<td>35</td>
<td>21,377</td>
</tr>
<tr>
<td>10255</td>
<td>0.333</td>
<td>0.2–0.6</td>
<td>10</td>
<td>36,292</td>
</tr>
<tr>
<td>10261</td>
<td>0.133</td>
<td>0–0.2</td>
<td>4</td>
<td>36,509</td>
</tr>
<tr>
<td>10267</td>
<td>0.067</td>
<td>0–0.2</td>
<td>2</td>
<td>50,619</td>
</tr>
<tr>
<td>10268</td>
<td>0.133</td>
<td>0–0.2</td>
<td>4</td>
<td>29,069</td>
</tr>
<tr>
<td>10269</td>
<td>0.10</td>
<td>0.05–0.2</td>
<td>3</td>
<td>45,891</td>
</tr>
<tr>
<td>10274</td>
<td>0.233</td>
<td>0.1–0.3</td>
<td>7</td>
<td>66,792</td>
</tr>
</tbody>
</table>

Geq: genome equivalents per milliliter of maternal blood.

aFor each sample, 12–21 amplifications were performed for the DSY1 locus. Amount shown is the mean value for amplification results in each sample.

bNumber of fetal progenitor cells expected in 30 ml of maternal blood based on the indicated mean values of genome equivalents.

cIncludes 8 out of 9 cases confirmed to have a male fetus and classified as correct detection with >2 positive amplifications for Y-chromosome sequences.

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Table 2. Correct detection of fetal gender in maternal blood specimens after RosetteSep™ enrichment and fluorescent in situ hybridization (FISH) analysis

<table>
<thead>
<tr>
<th>Fetal gender</th>
<th>Progenitor cell selection</th>
<th>CD45 negative cell selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male cases (n = 26)</td>
<td>10/19 (53%)</td>
<td>0/7 (0%)</td>
</tr>
<tr>
<td>Female cases (n = 37)</td>
<td>29/30 (97%)</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>Total (n = 63)</td>
<td>39/49 (80%)</td>
<td>7/14 (50%)</td>
</tr>
</tbody>
</table>
Because the differences in fetal cell detection between enrichment by the progenitor method and the CD45 depletion method were so dramatic, we wondered if the two methods selected different populations of cells. Ideally, evaluation of fetal blood would permit reliable analysis of the enriched cell population to identify cell types recovered by either of the two described enrichment methods. However, given the difficulty in obtaining sufficient volume of fetal blood for split specimen examination, we used cord blood as a surrogate for fetal cells. Figure 1 shows the result of flow cytometric analysis of CD34+ CD45+ cells (white blood cell progenitors) in (a); CD34+ CD33+ (myeloid progenitors and myeloid lineage cells) in (b); and in (c), CD14+ CD13+ cells (committed myeloid cells, monocytes and tissue macrophages). Results clearly show that the progenitor method enriches a distinct population of cells that are CD45+, CD33+, CD14+, CD13+, but CD34dim. This population is not enriched by CD45 depletion, suggesting there exists differential selection of fetal cells using the progenitor method. Preliminary results using flow cytometric analysis on maternal blood specimens have revealed similar results. Further studies are warranted to precisely identify the recovered fetal cell type using simultaneous immunohistochemical staining with FISH.

**Discussion**

Our detection rate of 53% in this pilot study suggests great promise for the RosetteSep™ progenitor enrichment method. Failure to achieve similar detection rates by FISH and real-time PCR could indicate that intact fetal cells are present but escape detection due to their relative rarity. With improved technology (i.e., automated microscope slide scanning), detection rates of 90% could be possible. Perhaps these promising results reflect simplistic and rapid methods that involve a single density gradient and centrifugation, limiting the potential for fetal cell loss. Moreover, unlike other enrichment procedures, the antibody cocktail is added to the whole blood prior to density separation to maximize labeling of all potential fetal cells. To test the reliability of the progenitor enrichment approach, we performed spiking experiments. When cord blood was serially diluted into adult blood followed by progenitor enrichment, we observed 15- to 20-fold enrichment (data not shown). Alternatively, greater detection by real-time PCR may indicate presence of cell-free DNA; however, this seems less likely because plasma was initially removed prior to antibody incubation and enrichment. The greatest amounts of cell-free fetal DNA are known to be found in plasma (2, 14), far more than present in intact cells.

Our observed 53% detection rate for male cells by FISH is equal or better to that found with far more laborious flow cytometry or MACS methods. Certainly there are many reports in the literature describing methods of enrichment with various levels of sensitivity. However, most involve relatively small number of cases with little or no follow-up. The National Institute of Health Fetal Cell Study Group has recently reported the largest study (15). We reported an overall sensitivity of 41.4% for euploid male cases and 74.4% for aneuploid cases. In the current study, 10 out of 81 maternal cases were subsequently found to include chromosomally abnormal (aneuploidy and structural aberrations) as well as twin and triplets fetuses. Four of these cases involved female fetuses. Of the six cases in which a Y-chromosome was present, two were enriched using the CD45 RosetteSep™ method and four processed using the described progenitor enrichment approach. No fetal cells were detected with CD45 depletion, while using the later method fetal cells were detected in two cases. Thus our overall detection of 53% by FISH using a simple enrichment procedure is probably greater than by detection using more difficult and tedious MACS- and FACS-based methods.

Evaluation of cord blood specimens suggests that the progenitor enrichment approach selects a distinct population of cells that are not present after CD45 depletion. The CD45 method is likely removing too many cells resulting in loss of most if not all target cells. Because a large fraction of the maternal sample is composed of white blood cells, removal of cells using the CD45 antibody can result in rare cell trapping and subsequent loss. In addition, it is certainly possible that a target fetal cell type, other than white blood cells (monocytes), express small amounts of CD45 antigen. Such cells could include the myeloid progenitors. Our results suggest merit in enriching for progenitor cells and in particular for cells of myeloid lineage (Table 1). Although fetal progenitor cells have also been shown to exist in first and second trimester maternal blood samples (1, 16–18), little effort has been made toward targeting these cells for non-invasive prenatal diagnosis. One reason is the fear that fetal cells such as lymphocytes or CD34+ cells could persist from prior pregnancies. If these cells exist, arising from persistent stem cells, interference with diagnosis of future pregnancies is possible. However, these persistent stem cells
would be rare and, hence, give rise to far fewer circulating fetal progenitor cells than would be expected to originate from an ongoing pregnancy. In addition, if the cells were committed myeloid cells they would be unlikely to persist. This conclusion is supported by our finding of only one false-positive case by FISH (n = 30) and none by real-time PCR (n = 9). Coata et al. (19) further demonstrated that cells from prior pregnancies were not detected when selection was specifically targeted to fetal CD34+ stem cells. However, future studies are warranted to precisely identify the type of fetal cells recovered with the progenitor enrichment approach.

Although not previously exploited for intact fetal cell enrichment and analysis, fetal progenitor cells have been used as a source for fetal cell expansion in culture. Results are controversial,
and expansion and detection of fetal cells have not been demonstrated in all studies (17, 18, 20–23). Less than salutary results probably reflect not only rarity of fetal progenitor cells, but specific enrichment strategies and culture conditions. Presence of large numbers of maternal cells may further hinder the growth potential of the far fewer fetal cells (28). Although usually a maximum of 7–11 fetal progenitor cells in 20 ml of maternal blood has been reported (16, 17), some have detected many fewer fetal cells (18). One reason for the discrepancy may be that estimations have been based on positive selection of CD34+ cells and MACS enrichment. Yet not all progenitor cells express CD34 antigen. Fetal cell loss may also have occurred or alternate fetal progenitor cell type(s) could have been selected against. Using a real-time PCR method of analysis, we estimated as many as 2–35 fetal progenitor cells could be present in 30 ml of maternal blood, albeit not all recoverable by manual analysis. Given that progenitor enriched-recovered cells are suitable for FISH, automated microscope analysis may allow for more efficient scoring of all recovered cells and thus higher sensitivity (24). The RosetteSep™ progenitor enrichment assay should therefore be capable of improvement for intact fetal cell analysis or expansion in culture.

Acknowledgements
We thank Dr Anthony Gregg and Audrey Burke, MS., for collection of cord blood and maternal blood specimens. The work has been supported by NIH/NICHD contract no. HD43203

References