Trophoblast retrieval and isolation from the cervix (TRIC) for noninvasive prenatal screening at 5 to 20 weeks of gestation

Jay M. Bolnick, M.D.,[a] Brian A. Kilburn, B.S.,[a] Swati Bajpayee, B.S.,[a] Nitya Reddy, B.S.,[a] Roohi Jeelani, M.D.,[a] Barbara Crone, R.N.,[a] Neil Simmerman, M.D.,[a] Manivinder Singh, M.D.,[a] Michael P. Diamond, M.D.,[b] and D. Randall Armant, Ph.D.[a,c,d]

[a] Department of Obstetrics and Gynecology, C. S. Mott Center for Human Growth and Development, Wayne State University, Detroit, Michigan; [b] Department of Obstetrics and Gynecology, Georgia Regents University, Augusta, Georgia; [c] Anatomy and Cell Biology, C.S. Mott Center for Human Growth and Development, Wayne State University, Detroit, Michigan; and [d] Program in Reproductive and Adult Endocrinology, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

Objective: To use trophoblast cells accumulating in the endocervical canal at the beginning of pregnancy for noninvasive prenatal testing.
Design: Prospective, double-blinded test for fetal gender.
Setting: Academic medical center.
Patient(s): Fifty-six women with singleton pregnancies at gestational age 5–20 weeks.
Intervention(s): Isolation of fetal cells from resident maternal cells in endocervical specimens using anti-human leukocyte antigen G coupled to magnetic nanoparticles; cell phenotyping immunofluorescently with a panel of trophoblast subtype-specific proteins; DNA integrity assessment with terminal dUTP nick-end labeling (TUNEL); and polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) to detect sex chromosomes in individual cells.
Main Outcome Measure(s): Trophoblast phenotype, TUNEL index, and percentage male cells.
Result(s): The women were given a routine Papanicolaou test; fetal genders were verified from medical records. Recovery after immunomagnetic isolation averaged 746 ± 59 cells across gestational age, with 99% expressing chorionic gonadotropin, whereas the depleted cell fraction expressed none. The isolated cells had an extravillous trophoblast phenotype and intact nuclear DNA (>95%). Fetal gender was determined in 20 specimens without error by PCR. The FISH analysis of isolated cells from male specimens validated their fetal origin.
Conclusion(s): Noninvasive prenatal testing is feasible beginning at a gestational age of 5 weeks. (Fertil Steril® 2014;102:135–42. ©2014 by American Society for Reproductive Medicine.)
Key Words: Endocervical canal, fetal gender, prenatal testing, single-cell analysis, trophoblast

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Prenatal diagnostic testing provides vital medical information for the management of both maternal and fetal well-being during pregnancy. Invasive procedures to procure fetal DNA, including chorionic villous sampling (CVS) at week 10 and amniocentesis at week 14 (1), have long been the standard of care for definitive prenatal genetic diagnosis. Cell-free fetal DNA obtained from maternal plasma offers a promising noninvasive approach that relies on next-generation sequencing and

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Reprint requests: D. Randall Armant, Ph.D., Mott Center for Human Growth & Development, Wayne State University, 275 East Hancock Street, Detroit, Michigan 48201–1405 (E-mail: d.armant@wayne.edu).

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bioinformatics to distinguish small fragments of fetal DNA from a much greater fraction of maternal DNA (2). However, it would be advantageous to acquire the entire genome by noninvasive collection of intact fetal cells.

Since the early 1970s (3), investigators have attempted to use minimally invasive methods to secure extraembryonic cells from the reproductive tract for genetic testing and assessment of placental pathology (4). Fetal cells have been obtained with varying success from the endocervical canal or uterine cavity by aspiration, endometrial biopsy, and lavage (4). Previous studies reported that cervical mucus retrieved from pregnant women provides cells identified as trophoblast in up to 70% of attempts, depending on the collection method (5–8). In 2005, a comparison of intrauterine lavage and cytobrush sampling of endocervical mucus (9) showed that intrauterine lavage yields trophoblast cells more consistently but is considerably more invasive. Others have obtained quite promising results with the less intrusive cytobrush (10).

We reported finding trophoblast cells in over 95% of specimens collected from pregnant patients using a commercial Papanicolaou test kit with a cytobrush (11). There were no adverse medical outcomes in that study, and an equivalent number of fetal cells was obtained at gestational ages (GA) of 6–14 weeks. Trophoblast cells were identified by immunohistochemistry using antibody against the fetal-specific protein, human leukocyte antigen G (HLA-G), which is expressed by human extravillous trophoblast cells and is not expressed in adult tissues of the cervix or uterine cavity (12, 13). It is interesting that the number of trophoblast cells recovered was significantly decreased in the case of ectopic or blighted ovum pregnancies, suggesting a potential use of this approach to distinguish adverse pregnancy outcomes (11).

Noninvasive retrieval of fetal cells from the endocervical canal is a promising approach for obtaining fetal DNA early in gestation for prenatal genetic screening. Acquired cells have been evaluated by fluorescent in situ hybridization (FISH) for the presence of the Y chromosome (14). Others have assessed single gene disorders and fetal genotype (15, 16). However, the usefulness of these trophoblast cells has been greatly hampered by their minimal presence in an overwhelming excess of maternal cervical cells. This report demonstrates the utility of trophoblast retrieval and isolation from the cervix (TRIC) during weeks 5–20 of pregnancy and establishes the subtype of trophoblast cells captured from the endocervical canal. Trophoblast cells retrieved from the endocervical canal were for the first time isolated free of maternal cells using anti-HLA-G coupled to magnetic nanoparticles and were assessed for homogeneity based on fetal-specific parameters, including expression of the β-subunit of human chorionic gonadotropin (β-hCG) and, in pregnancies with a male fetus, assessment of X and Y chromosomes by FISH.

MATERIALS AND METHODS

Patient Selection and Ethical Approval

Fifty-six patients receiving initial prenatal care after spontaneous conception or infertility treatment were consented for collection of endocervical specimens at Wayne State University and Detroit Medical Center facilities, including Hutzel Women's Hospital, Sinai-Grace Hospital, and the Southfield Clinic of Wayne State University Physicians Group. The Institutional Review Board of Wayne State University approved the study, with each individual providing written informed consent prior to participation. The inclusion criteria specified pregnant women between the ages of 18 and 45, GA from 5 to 20 weeks. Exclusion criteria included women who were experiencing active vaginal bleeding and multiple gestations. GA was determined by the date of the last menstrual period and the first ultrasound.

Endocervical Sampling

Endocervical sampling was conducted as described previously elsewhere (11). Briefly, the cervical specimens were collected with a ThinPrep kit (Hologic) using a cytobrush. The cytobrush was rinsed into 20 mL of PreservCyt (Hologic) fixative solution, which was acidified to dissolve mucous and centrifuged at 400 $\times$ g for 5 minutes at 4°C. After washing the cells three times in 20 mL of phosphate buffered saline (PBS), the cells were brought to 10 mL with PBS at 4°C. An aliquot ($\sim$1 mL) of each processed specimen containing up to 200,000 cells was centrifuged onto a slide using a Shandon Cytospin 3 centrifuge (Thermo-Fisher) and labeled with 10 μg/mL of mouse anti-HLA-G (Clone 4H84, BD Biosciences; or Clone G233, Exbio) to estimate the content of trophoblast cells, as previously described elsewhere (11).

Immunomagnetic Isolation of Trophoblast Cells

Mouse anti-HLA-G antibody (5 μg) was incubated overnight at 4°C in 100 μL sterile PBS containing 20 μL of 250 nm magnetic nanoparticles conjugated to goat anti-mouse IgG (Clemente Associates). The nanoparticles were washed three times with PBS using a DynaMag-Spin magnet (Life Technologies). The processed endocervical cells were centrifuged and resuspended in 1.5 mL PBS, combined with the HLA-G-coupled nanoparticles, and incubated overnight at 4°C with mixing. The nonbound cells were collected after magnetic immobilization and three washings in PBS. A small aliquot of the isolated cell suspension was removed, labeled with 1 ng/mL of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), and the DAPI-labeled nuclei were counted by epifluorescence microscopy to determine the number of cells recovered in the isolate.

Twenty of the specimens were archived for gender analysis by polymerase chain reaction (PCR). While viewing with transmitted light under a Wild M3Z stereomicroscope (Leica Microsystems), we drew 20–50 isolated cells in a 10-μL drop into the 75-μm tip of a Stripper micropipetter (Origio MidAtlantic Devices) and sorted them into single cells that were placed individually into 0.2-mL PCR tubes (Molecular BioProducts) with approximately 4 μL of RNase-free water. These were frozen at −80°C.

Immunofluorescence Microscopy

Isolated HLA-G-positive cells and recovered HLA-G-depleted cells were prepared for immunofluorescence microscopy by

suspending groups of approximately 50 cells in 200 μL of PBS and centrifuging them onto a slide using the Shandon Cytospin 3 at 1500 RPM for 5 minutes. The slides were labeled overnight at 4°C with 10 μg/mL of primary antibody (see the list in Supplemental Table 1, available online). Controls were nonimmune IgG of the same species as the primary antibody (Jackson ImmunoResearch). Primary antibody was visualized using fluorescein isothiocyanate (FITC) conjugated secondary antibodies with a DAPI nuclear counterstain, as previously described elsewhere (11). Images of DAPI and FITC staining were captured by epifluorescence microscopy using a Leica DM IRB microscope and a Hamamatsu Orca cooled-chip digital camera for counting labeled and unlabeled cells.

DNA Fragmentation

Fragmented DNA was detected in isolated trophoblast cells from nine patients by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method with a DAPI counterstain, as previously described elsewhere (17). The HTR-8/SVneo human cytotrophoblast cells were cultured in 96-well plates and exposed to vehicle (control) or 50 mM ethanol for 60 minutes, as previously described elsewhere (18). The cultured cells were tested in triplicate wells in six experiments. The fraction of TUNEL/DAPI labeled nuclei (TUNEL index) was determined by cell counting from micrographic images.

FISH

Isolated cell fractions centrifuged onto slides were analyzed at the Detroit Medical Center Cytogenetics Laboratory by FISH (19). The X and Y chromosomes were visualized using CEP X (DXZ1; Xp11.1-q11.1 alpha satellite) SpectrumOrange and CEP Y (DYZ1; Yq12 satellite III) SpectrumGreen probes (Vysis), respectively.

Single-Cell PCR

The PCR tubes containing single, isolated trophoblast cells or BJ foreskin fibroblast cells (American Type Culture Collection [ATCC]) fixed in PreservCyt (Cytyc Corporation) were heated to 100°C for 10 minutes and cooled to room temperature. Human male DNA was isolated from BJ cells using a DNeasy blood and tissue kit (Qiagen), and 20 ng was amplified for X and Y PCR controls. Ten microliters of HotStarTaq Plus Master Mix (Qiagen) was added to each tube. Previously described (20) forward and reverse primers for a 459 bp sequence in *DMD* exon 19 and a 351-base pair (bp) sequence in the *SRY* gene were reconstituted in Tris-EDTA buffer and added to a final concentration of 10 μM. The DNA was amplified using an Eppendorf Mastercycler. Initial activation at 95°C for 5 minutes was followed by 35 cycles of denaturing for 1 minute at 94°C, annealing for 1 minute at 58°C, and extension for 1 minute at 72°C, with a final extension for 10 minutes at 72°C. An 8-μL aliquot of each reaction product was combined with 2 μL of loading dye containing ethidium bromide, separated by electrophoresis on a 1% agarose gel and photographed on an ultraviolet transilluminator. Bands were compared with a premixed 100 bp DNA ladder (Life Technologies).

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). Correlations were determined by regression and Pearson correlation analysis with SPSS statistical software (IBM). The TUNEL index values were compared by analysis of variance (ANOVA) with a two-tailed *t*-test for post-hoc analysis. $P<.05$ was considered statistically significant.

RESULTS

Isolation of Trophoblast Cells

Fifty-six endocervical specimens were obtained from pregnant women with fetuses ranging in GA between 5 and 20 weeks (details in Supplemental Table 2, available online). The mean maternal age was 27.5 ± 6.7 years; 15 women were nulliparous, and 41 were multiparous. Eight delivered preterm and 14 by cesarian delivery. Estimates of total trophoblast cell number determined by immunohistochemical analysis of HLA-G expression in cytospun aliquots indicated that the specimens contained an average of 1,275 ± 137 fetal cells. Counts of all trophoblast cells recovered after immunomagnetic isolation averaged 912 ± 55 cells per specimen. On average, the recovery was 106% ± 13% (range: 10%–550%) of the original estimates, which were subject to high error due to the small number of cells actually counted.

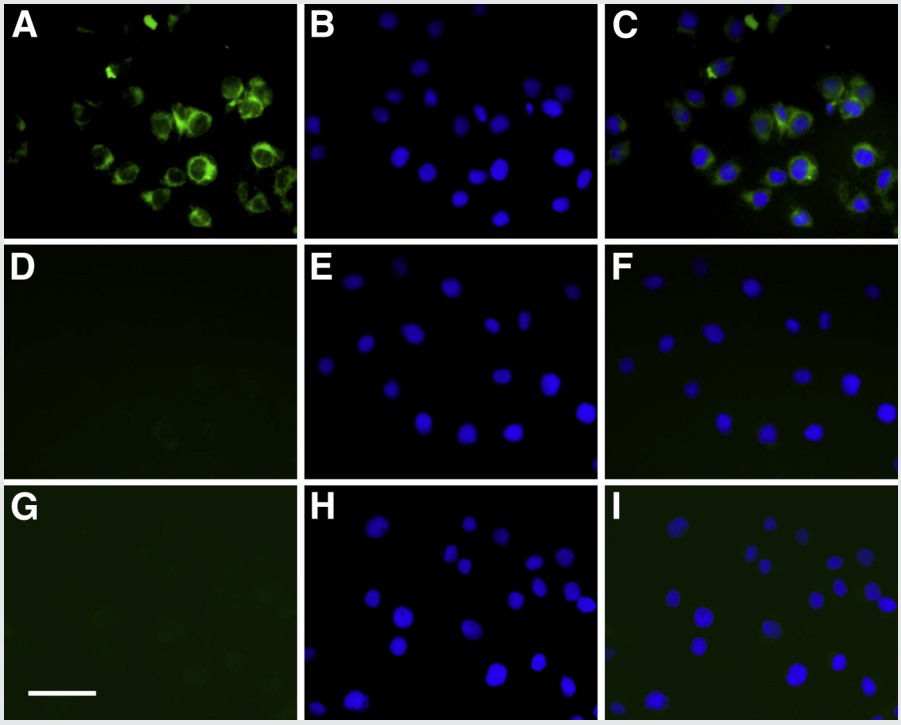
Trophoblast cells were obtained by TRIC as readily at week 5 of gestation as they were later in the pregnancy (Supplemental Fig. 1, available online). Regression analysis comparing GA with the number of trophoblast cells recovered produced a statistically nonsignificant ($P=.72$) Pearson correlation coefficient of 0.048. Immunofluorescence for β-hCG revealed extensive expression among the isolated cells (Fig. 1A). The average percentage of cells expressing β-hCG among the magnetically bound cells was 99% (range: 94%–100%; Supplemental Table 2), and none of the cells examined in the excluded, nonbound fraction were β-hCG positive (see Fig. 1D).

Probing for the X and Y chromosomes by FISH in cells obtained from pregnancies that produced a male fetus provided additional evidence that TRIC effectively isolates fetal cells. Six specimens from pregnancies that produced the birth of a male or displayed a Y chromosome by PCR determination were hybridized with probes for the X and Y chromosomes. A total of 278 cells out of 280 examined were XY (quantified in Supplemental Table 3, available online). Specimens from pregnancies that produced a female fetus were labeled only with the X probe. Only a single X label was detected in a few cells. Examples of FISH staining of TRIC specimens with male or female fetuses are shown in Figure 2.

Phenotype of Isolated Trophoblast Cells

To further establish that the isolated cells were trophoblast and determine their specific phenotype, immunofluorescence microscopy was used to examine expression of marker proteins (21–26) for human villous cytotrophoblast,

FIGURE 1



Expression of β-hCG in cells isolated using anti-HLA-G coupled to magnetic nanoparticles. Bound (A–C and G–I) and the excluded nonbound (D–F) cells were labeled with anti-β-hCG (A–F) or nonimmune IgG (G–I) and examined by immunofluorescence microscopy. Images of secondary antibody conjugated to FITC (*green*) appear in the left panels and matching fields of nuclei counterstained with DAPI (*blue*) appear in the center panels. Overlays of the FITC and DAPI stain appear in the right panels. Size bar is 50 μm.

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syncytiotrophoblast, and extravillous trophoblast subtypes (Table 1). Immunomagnetically isolated cells expressed β-hCG, placental lactogen (CSH1), and cytokeratin 7 (KRT7), which are all expressed throughout the trophoblast lineage (21–23). However, the isolated cells were not reactive with antibodies against pregnancy-specific beta-1-glycoprotein 1 (PSG1), integrin-α6 (ITGA6), or E-cadherin (CDH1), which are all expressed predominantly in trophoblast populations of the chorionic villi (23, 24). Five proteins expressed by extravillous trophoblast cells (23, 25, 26), VE-cadherin (CDH5), platelet endothelial cell adhesion molecule precursor (PECAM1), integrin-α1 (ITGA1), matrix metalloproteinase 9 (MMP9), and HLA-G, were all expressed in cells obtained by TRIC.

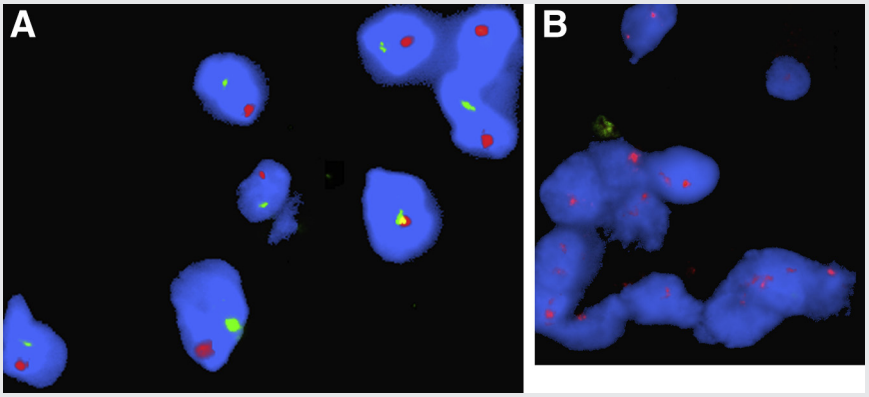
Integrity of DNA from Isolated Trophoblast Cells

Potentially, TRIC could provide fetal DNA for prenatal diagnosis. The integrity of the trophoblast DNA was assessed for gross fragmentation by TUNEL. We previously established that apoptosis is significantly increased in the human trophoblast cell line HTR-8/SVneo after exposure for 1 hour to 50 mM ethanol (18), and repeated this treatment for comparison with TUNEL in trophoblast cells (n = 545) isolated from nine endocervical specimens (Supplemental Fig. 2, available online). Although ethanol exposure increased the TUNEL index of HTR-8/SVneo cells from 0.050 to 0.12 ($P<.00001$), the TUNEL index of trophoblast cells obtained by TRIC was comparable to the control cells, at 0.035 ($P=.17$).

Gender Determination Using Isolated Trophoblast Cells

To establish that trophoblast cells isolated from transcervical specimens were a valid source of fetal DNA for single-cell genetic analysis, multiplex PCR was conducted with replicates of individual frozen cells from 20 patient specimens using primers for *DMD* and *SRY* to detect the presence of an X (control) or Y chromosome, respectively. Male fetuses were readily identified by amplification of two bands, and the trophoblast cells from female fetuses produced only the X band (Fig. 3). For positive controls, the PCR analysis included *DMD*, *SRY*, or multiplex amplification of both genes using isolated foreskin fibroblast DNA or a single foreskin fibroblast. The fetal gender of all 20 pregnancies was correctly determined, as verified by patient records (Supplemental Table 4, available online). There were 9 female and 11 male fetuses among the 20 specimens examined. The ability to distinguish *SRY* amplicons in specimens with a male fetus ranged from 25 of 28 (89.3%) to 10 of 10 (100%), providing a clear result in an average of 96.5% of the PCR reactions. Detection of the *DMD* reaction product ranged from 25 of

FIGURE 2



FISH for X and Y chromosomes in trophoblast cells obtained by TRIC. Trophoblast cells in TRIC samples from pregnancies with a male (A) or female (B) fetus were labeled with probes for the DYZ1 satellite III on the Y chromosome (*green*) or the DXZ1 alpha satellite on the X chromosome (*red*). Nuclear chromatin is labeled with DAPI (*blue*). A pair of sex chromosomes is labeled over most nuclei. The green signal in B is due to nonspecific labeling of debris not associated with a nucleus.

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30 (83.3%) to 25 of 25 (100%), with 96.2% of the reactions successful on average. Overall, there were no visible bands produced by PCR in 14 of 270 cells evaluated (5.2%). The lower panel in Figure 3 contains two examples of failed amplification from archived fetal cells as well as some weakly amplified products. The PCR-determined genders of individual cells were homogeneous within every specimen.

DISCUSSION

It has long been understood that trophoblastic elements of the placenta accumulate in the endocervical canal and can be recovered by a number of methods with variable success (4). Noninvasive capture of extravillous trophoblast cells could be used for prediction of obstetric disorders and provide fetal DNA for prenatal genetic diagnosis if the fetal cells can be accurately identified and efficiently isolated. HLA-G has proven to be a reliable protein marker to identify trophoblast cells collected from the cervix (11, 27, 28). Other monoclonal antibodies that distinguish trophoblast cells from adult tissues have also been used (29). Laser capture of HLA-G labeled cells has been combined with quantitative PCR to determine fetal gender and identify fetal-specific alleles by short terminal repeat analysis in a small number of specimens (27). Others have used microdissection to isolate cells based on trophoblast markers to perform allelic profiling by multiplex PCR (29, 30), again with the limitations imposed by manual cell separation. Although this approach could provide extremely useful information to clinicians, it is not a robust method due to the extreme excess of maternal cells from which fetal cells must be distinguished for accurate analysis.

We found that TRIC efficiently, rapidly provided adequate numbers of trophoblast cells for prenatal testing with minimal contamination by maternal cells. Approximately 500–1,500 trophoblast cells were obtained from each patient specimen, and 95%–100% of the cells expressed β-hCG, a trophoblast-specific protein. In specimens with a male fetus, FISH revealed that over 99% of isolated cells had an XY genotype, verifying that they were indeed of fetal origin. The utility of TRIC for prenatal testing was demonstrated in a multiplex PCR assay that distinguished male and female fetal cells with a high degree of confidence. Compared to approaches that use cell-free fetal DNA in maternal plasma (2), TRIC captures the entire fetal genome with each cell, does not require massively parallel sequencing and bioinformatics to distinguish fetal and maternal genetic information, and can be monitored for maternal cell contamination using β-hCG immunofluorescence microscopy as a quality control measure. The observed paucity of nick end labeling in the isolated trophoblast cells suggests that their DNA is unfragmented. Furthermore, TRIC is robust at a GA of 5 weeks, as reported elsewhere (31) and

TABLE 1

Expression of trophoblast markers in cells isolated by TRIC.

Protein	Isolated cells[a]	vST[b]	vCT[b]	EVT[b]	Ref.[b]
CG β subunit	+	+	+	+	(21)
KRT7	+	+	+	+	(22, 23)
CSH1	+	+	–	+	(21, 22)
PSG1	–	+	–	–	(24)
ITGA6	–	+	+	–	(23)
CDH1	–	+	+	+/–	(23)
CDH5	+	–	–	+	(23)
PECAM1	+	–	–	+	(23)
ITGA1	+	–	–	+	(23)
MMP9	+	–	+	+	(25)
HLA-G	+	–	–	+	(26)

[a] Groups of approximately 50 anti-HLA-G-binding cells isolated with magnetic nanoparticles were examined by immunofluorescence microscopy and their reactivity to each antibody is shown. Reactivity was all or none for each protein tested.
[b] The known expression of each protein in villous syncytiotrophoblast (vST), villous cytotrophoblast (vCT) and extravillous trophoblasts (EVT), as well as the reference sources, appears in the other columns.

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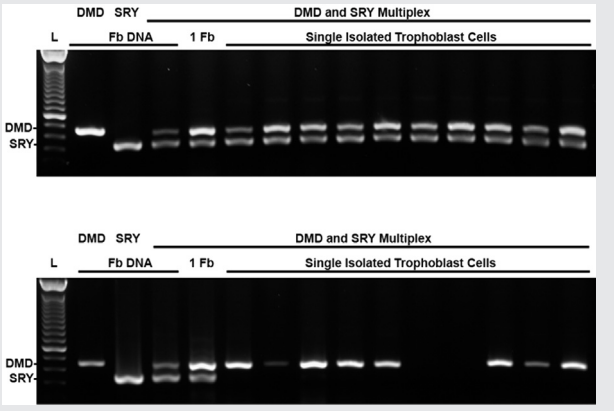
FIGURE 3



Single-cell PCR for fetal gender determination. Multiplex PCR for *DMD* and *SRY* on the X and Y chromosomes, respectively, was used for gender determination with individual fetal cells obtained by TRIC from two patients. Lanes in each panel include a 100-bp ladder (L), followed by control DNA isolated from foreskin fibroblasts (Fb) assayed for *DMD*, *SRY*, or both genes. Next is a single fixed Fb (1 Fb) assayed for both genes. The last 10 lanes are multiplex amplifications of *DMD* and *SRY* in individual trophoblast cells. All 10 cells from the patient in the upper panel amplified both *DMD* and *SRY*, indicating a male fetus. In the lower panel, only *DMD* amplified, indicating a female fetus. The lower panel contains examples of weak and failed amplifications. Results for 20 patients are summarized in Supplemental Table 4 (available online).

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confirmed here, and provides similar numbers of fetal cells through a GA of 20 weeks. The findings of this investigation indicate TRIC to be a powerful approach for prenatal testing, but it was limited by a small sample size and additional trials will be required to establish clinical reliability of TRIC for each application.

Trophoblast phenotype assessed with a panel of antibodies (see Table 1) confirmed that cells isolated with the anti-HLA-G antibody were indeed trophoblast, based on their expression of KRT7, CSH1, and β-hCG, which are expressed in both villous and extravillous trophoblast but not in other placental or decidual cell types. Furthermore, the isolates lacked reactivity with antibodies against proteins expressed by villous trophoblast (PSG1, ITGA6, and CDH1) and expressed several proteins associated with extravillous trophoblast populations (HLA-G, ITGA1, CDH5, PECAM1, and MMP9). CDH1 is expressed at low levels in interstitial trophoblasts and is completely absent during intravascular invasion (23), consistent with the idea that the cells isolated from the endocervical canal were deeply invasive trophoblasts. Therefore, it is hypothesized that invasive extravillous trophoblast cells migrate as far as the endocervical canal during the initial weeks of the first trimester where they can be recovered noninvasively. However, there is immunological evidence that villous trophoblast cells are also present in the cervical mucus (29). By selecting HLA-G-expressing cytotrophoblast cells, multinucleated syncytia and apoptotic villous debris were excluded from the material obtained by TRIC. HLA-G protein is first expressed in trophoblast cells of the anchoring villi that have differentiated into invasive extravillous trophoblasts (26).

Fetal gender, as confirmed by birth records, was determined without error in all 20 TRIC specimens examined. Preimplantation genetic diagnostics have been developed for single-cell PCR analysis of a wide spectrum of single-gene disorders (32). TRIC coupled with these molecular approaches would be directly applicable for screening within the general population of pregnant patients at earlier GA and with less risk than CVS or amniocentesis. Couples who conceive without IVF and are known to be carriers of genetically linked disorders could be provided fetal genetic information very early in pregnancy through TRIC. Within a month of fertilization, TRIC can provide adequate numbers of cells for prenatal analysis without significant risk to the pregnancy. Indeed, a Papanicolaou test using the cytobrush is not contraindicated during pregnancy (33).

Because TRIC provides hundreds of fetal cells, multiple replicates of single cells can be assayed by PCR, significantly reducing errors caused by maternal cell contamination or allele drop out. The probability (P) of selecting a maternal cell was less than 0.1 (non-β-hCG expressing cells were 0–5%) and decreased exponentially with each additional replicate (N), where $P<10^{-N}$. No evidence of maternal contamination was found among the 11 TRIC specimens examined from pregnancies with a male fetus because none of the replicate cells produced a single band rather than an X/Y duplex. Amplification failures (5.2% overall) were only found that resulted in loss of both bands when they occurred when a male fetus was present, suggesting that they were due to either failed PCR amplification or loss of the trophoblast cell during archiving.

A complication not encountered among the patients studied was multiple gestation, which could produce genetically distinct fetal cells. It should be possible to identify and separately assess multiple fetuses by TRIC when single-cell methods are conducted in tandem with DNA genotyping using short tandem repeat sequences or single-nucleotide polymorphisms. It is possible that, in multiparous women, trophoblast cells from previous pregnancies could be obtained by TRIC along with those of the current pregnancy. Fetal cells can persist in maternal blood for years, although their abundance is quite low and they are unlikely to include trophoblast (34, 35). Experiments conducted with rats indicate that trophoblast clearance from the uterus, and presumably the cervix, is complete in a few days (36). After elimination of the chorion, either by delivery or preterm extraction, extravillous trophoblast cells are absent by postpartum day 4. Trophoblast clearance from the uterine wall is also observed in humans, requiring about 10 days (37, 38). Persistence of extravillous trophoblast is associated with postpartum hemorrhaging due to subinvolution of the uteroplacental arteries (37), suggesting that their removal from the placental bed is a normal step in reconstituting the maternal vasculature.

Single-cell analysis by microarray and quantitative PCR has been successfully used for chromosome analysis (39–42) and could be adapted for TRIC. It is possible that with next-generation sequencing TRIC could be used to deduce the entire genomic sequence of the fetus, as has been reported

using cell-free fetal DNA (43, 44). A more prominent role for genomewide sequencing can be anticipated as personalized medicine becomes standard care and interpretation of the human genome is sufficiently expanded to identify more fetal and perinatal pathologies (45).

In summary, we have found that immunomagnetic isolation of trophoblast cells from endocervical specimens is an efficient and noninvasive method of procuring fetal DNA and trophoblast tissue at the beginning of the first trimester for prenatal analysis. It remains to be established whether cell collection is as robust before a GA of 5 weeks and what the limitations are for prenatal testing and experimental investigation using this novel approach.

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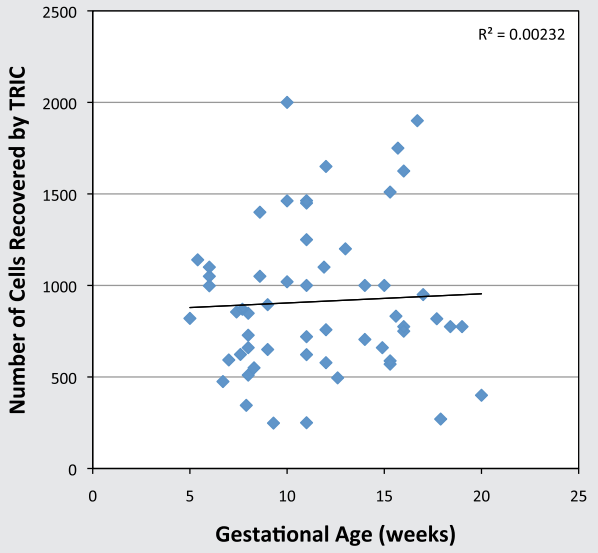
SUPPLEMENTAL FIGURE 1



Recovery of HLA-G-positive cells according to gestational age. The number of fetal cells recovered by TRIC from individual specimens are shown with respect to gestational age at the time of collection. A linear regression line is drawn through the values and the R^2 value is shown in the upper right corner.

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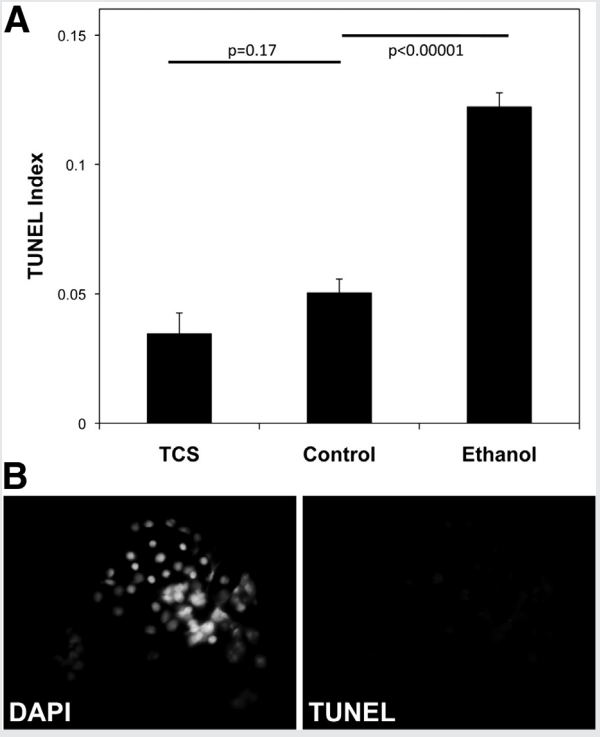
SUPPLEMENTAL FIGURE 2



DNA fragmentation analysis. (A) TUNEL was assessed in trophoblast cells obtained by TRIC and HTR-8/SVneo cytotrophoblast cells that were either untreated (control) or exposed to 50 mM ethanol for 1 hour (ethanol). Total cell number, determined by DAPI colabeling, and the number of TUNEL-positive cells were used to calculate the TUNEL index for trophoblast cells (n = 545) isolated from nine transcervical specimens (TCS), and for HTR-8/SVneo control (n = 1,394) and ethanol-exposed (n = 1,366) cells. ANOVA was performed, and comparisons to the control in post-hoc two-tailed *t*-tests are indicated above the bars. (B) Example of a field of cells obtained by TRIC showing DAPI nuclear staining in the left panel and TUNEL in the right panel.

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SUPPLEMENTAL TABLE 1

Antibodies against human proteins used to characterize trophoblast cells.

Antigen	Clone	Species	Company
HLA-G	4H84	Mouse	BD Biosciences
β-CG	5H4-E2	Mouse	Thermo Scientific
Cytokeratin7 (KRT7)	OV-TL 12/30	Mouse	DAKO
Placental lactogen (CSH1)	658230	Mouse	R&D Systems
PSG1	ag2468	Rabbit	Proteintech Group
Integrin α6A (ITGA6A)	1A10	Mouse	Millipore
E-Cadherin (CDH1)	32A8	Mouse	Cell Signaling Technology
VE-Cadherin (CDH5)	2158	Mouse	Cell Signaling Technology
PECAM1 (CD31)	89C2	Mouse	Cell Signaling Technology
Integrin α1 (ITGA1)	5E8D9	Mouse	Millipore
MMP9	4H3	Mouse	R&D Systems

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SUPPLEMENTAL TABLE 2

Trophoblast content, recovery, and purity.

Gestational age (wk.d)	No. of HLA-G+ cells[a]	No. of isolated cells[b]	HLA-g+ cell recovery (%)	β-hCG reactive[c] (%)
5	1,025	820	80	100
5.3	1,313	1,140	87	100
6	2,250	1,050	47	96
6	1,200	1,100	92	97
6	609	998	164	95
6.5	4,775	475	10	97
7	575	593	103	100
7.3	677	855	126	99.6
7.4	842	623	74	98
7.5	314	870	277	100
7.6	1,108	345	31	98.8
8	593	510	86	100
8	1,045	728	70	98.9
8	609	660	108	100
8	788	848	108	99
8.2	100	550	550	97
8.4	1,825	1,050	58	100
8.4	525	1,400	267	95
9	725	895	123	96
9	1,425	650	46	97
9.2	1,260	248	20	99
10	1,700	2,000	118	98
10	2,222	1,462	66	97.8
10	1,095	1,020	93	100
11	1,175	1,000	85	100
11	175	250	143	96
11	1,450	1,450	76	100
11	1,250	1,250	74	100
11	581	720	124	99.3
11	1,027	622	61	100
11	529	1,463	277	98.4
11.6	225	1,100	489	100
12	1,475	1,650	111	94
12	850	578	68	98.9
12	879	758	86	100
12.4	1,109	495	45	96.4
13	1,400	1,200	86	100
14	1,500	1,000	67	98
14	820	705	86	100
14.6	466	660	142	99
15	5,175	1,000	19	100
15.2	1,900	588	31	98
15.2	1,000	1,510	151	100
15.2	570	570	100	99.5
15.4	2,467	832	34	99.2
15.5	1,225	1,750	110	98
16	1,850	1,625	88	97
16	1,725	750	43	100
16	25	775	31	97
16.5	1,750	1,900	109	100
17	4,000	950	24	97
17.5	1,009	818	81	99.6
17.6	239	270	113	99.6
18.3	1,225	775	63	94
19	3,250	775	24	100
20	500	400	80	100
Mean ± SE	1,275 ± 137	912 ± 55	106 ± 13	98.5 ± 0.23

[a] TRIC specimens obtained from patients at the indicated gestational ages were examined by immunohistochemistry for the presence of HLA-G and the total number of HLA-G-positive cells in each specimen was estimated.
[b] Anti-HLA-G-binding (HLA-G+) cells were isolated with magnetic nanoparticles and the total number of cells recovered from each specimen was estimated from cell counts.
[c] Immunofluorescence microscopy was used to determine the number of isolated cells expressing β-CG/total cells examined, with the percentage expressing β-CG calculated by counting at least 50 cells.

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SUPPLEMENTAL TABLE 3

FISH analysis of cells obtained by TRIC.

No. of cells examined	XX	XY	XO	PCR gender determination	Gender from birth record
46	0	45	1	M	Boy
56	0	55	1	M	Boy
50	0	50	0	M	Boy
53	0	53	0	M	Boy
35	0	35	0	M	Boy
32	0	32	0	M	Loss at 16 wk
41	37	0	4	F	Girl
52	45	0	7	F	ND
21	15	0	6	F	ND

Note: Cells were visualized by epifluorescence microscopy to observe DAPI labeled nuclei, Spectrum Orange-labeled X chromosome probe and Spectrum Green-labeled Y chromosome probe. The number of cells scored as XX and XY is shown, as well as the number of cells in which only a single X signal was detected (XO). Verification of gender by either PCR or birth of the fetus is indicated in the last two columns. ND = not determined. Patients declined ultrasonography determination of gender and had not delivered at the time of writing.

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SUPPLEMENTAL TABLE 4

Fetal gender determination.

Gestational age (wk.d)	Chromosome detected (%)[a] X	Y	Gender verification[b]
5.3	10/10 (100)	0/10 (0)	Female
6.0	25/30 (83.3)	0/30 (0)	Female
7.6	25/25 (100)	0/25 (0)	Female
8.0	10/10 (100)	0/10 (0)	Female
10.0	10/10 (100)	0/10 (0)	Female
12.0	10/10 (100)	0/10 (0)	Female
12.0	9/10 (90)	0/10 (0)	Female
12.4	10/10 (100)	0/10 (0)	Female
17.6	10/10 (100)	0/10 (0)	Female
7.3	5/5 (100)	5/5 (100)	Male
7.5	10/10 (100)	10/10 (100)	Male
8.0	25/28 (89.3)	25/28 (89.3)	Male
9.2	10/10 (100)	10/10 (100)	Male
10.0	10/10 (100)	10/10 (100)	Male
11.0	10/10 (100)	10/10 (100)	Male
12.0	24/26 (92.3)	24/26 (92.3)	Male
14.0	6/6 (100)	6/6 (100)	Male
14.6	18/20 (90)	18/20 (90)	Male
15.2	9/10 (90)	9/10 (90)	Male
17.5	10/10 (100)	10/10 (100)	Male

[a] Cells obtained by TRIC were assayed by single-cell, multiplex PCR for the X and Y chromosome markers *DMD* and *SRY*, respectively. Five to thirty replicate cells from each patient were examined to determine the presence or absence of each chromosome, presented as the number of cells that produced an amplicon/total cells examined.
[b] Genders were verified from medical or birth records.

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