Nondestructive Identification of Rare Trophoblastic Cells by Endoplasmic Reticulum Staining for Noninvasive Prenatal Testing of Monogenic Diseases

Yifang Huang, Bo Situ, Liping Huang, Yingsi Cao, Hong Sui, Xinyi Ye, Xiujuan Jiang, Aifen Liang, Maliang Tao, Shihua Luo, Ye Zhang, Mei Zhong, and Lei Zheng*

Noninvasive prenatal detection of monogenic diseases based on cell-free DNA is hampered by challenges in obtaining a sufficient fraction and adequate quality of fetal DNA. Analyzing rare trophoblastic cells from Papanicolaou smears carrying the entire fetal genome provides an alternative method for noninvasive detection of monogenic diseases. However, intracellular labeling for identification of target cells can affect the quality of DNA in varying degrees. Here, a new approach is developed for nondestructive identification of rare fetal cells from abundant maternal cells based on endoplasmic reticulum staining and linear discriminant analysis (ER-LDA). Compared with traditional methods, ER-LDA has little effect on cell quality, allowing trophoblastic cells to be analyzed on the single-cell level. Using ER-LDA, high-purity of trophoblastic cells can be identified and isolated at single cell resolution from 60 pregnancies between 4 and 38 weeks of gestation. Pathogenic variants, including –SEA deletion mutation and point mutations, in 11 fetuses at risk for α- or β-thalassemia can be accurately detected by this test. The detection platform can also be extended to analyze the mutational profiles of other monogenic diseases. This simple, low-cost, and noninvasive test can provide valuable fetal cells for fetal genotyping and holds promise for prenatal detection of monogenic diseases.

1. Introduction

Investigation of fetal genetic information in early pregnancy is of great importance for the prevention of genetic diseases.

Invasive procedures, such as amniocentesis (AC, 16 weeks of gestational age) and chorionic villous sampling (CVS, 10–12 weeks of gestational age), are the current gold standards for diagnosing fetal genetic diseases. However, these invasive procedures may increase the risk of fetal infection and miscarriage. Therefore, great efforts have been made to develop noninvasive approaches for prenatal genetic testing. Cell-free fetal DNA (cfDNA) based testing has high sensitivity and specificity in detecting common fetal chromosomal aneuploidies and has widely being implemented in clinical practice. However, cfDNA based assays for the detection of monogenic diseases, a category of diseases caused by point mutations or micro-deletions in pathogenic genes, are limited by their complicated steps of next-generation sequencing (NGS) and parental haplotyping. Furthermore, the highly fragmented nature (~200 bp) and low fetal fraction (with medians of 10–15%) of cell-free DNA in the maternal circulation may lead to unreliable results in fetal genotyping. Intact fetal cells for prenatal testing have attracted a great deal of attention. Pure fetal genomic DNA
Early pregnancy Pap samples ER-LDA analysis Single cell isolation Rare cell analysis Mutation analysis

Relative fluorescence intensity

ER-tracker Epithelial cell Epithelial cell Epithelial cell

Trophoblastic cell Trophoblastic cell Trophoblastic cell

The predictive probability of single cells for being a trophoblastic cell

Actual

Trophoblastic cells Epithelial cells White blood cells

ER-staining Immunofluorescence

Target sequence coverage (%)

| ER-staining | Immunofluorescence |
|-------------|-------------------|
| P1         | P11               |
| P2         | P12               |
| P3         | P13               |
| P4         | P14               |
| P5         | P15               |
| P6         | P16               |
| P7         | P17               |
| P8         | P18               |
| P9         | P19               |
| P10        | P20               |
| P11        | P21               |
| P12        | P22               |
| P13        | P23               |
| P14        | P24               |
| P15        | P25               |
| P16        | P26               |
| P17        | P27               |
| P18        | P28               |
| P19        | P29               |
| P20        | P30               |
| P21        | P31               |
| P22        | P32               |
| P23        | P33               |
| P24        | P34               |
| P25        | P35               |
| P26        | P36               |
| P27        | P37               |
| P28        | P38               |
| P29        | P39               |
| P30        | P40               |
| P31        | P41               |
| P32        | P42               |
| P33        | P43               |
| P34        | P44               |
| P35        | P45               |
| P36        | P46               |
| P37        | P47               |
| P38        | P48               |
| P39        | P49               |
| P40        | P50               |
| P41        | P51               |
| P42        | P52               |
| P43        | P53               |
| P44        | P54               |
| P45        | P55               |
| P46        | P56               |
| P47        | P57               |
| P48        | P58               |
| P49        | P59               |
| P50        | P60               |

0.00 0.20 0.40 0.60 0.80 1.00
can be obtained from individual fetal cells, allowing the determination of fetal genotype without statistical assessment. Furthermore, fetal cells with the entire fetal genome can provide fetal DNA with high integrity, making them suitable for use in the analysis of single nucleotide variations. In recent decades, many studies have focused on isolating circulating fetal cells from maternal peripheral blood. However, these efforts have had limited success because it is challenging to find extremely rare fetal cells among abundant maternal blood cells (>6 fetal cells and 10⁷ maternal cells per mL of blood). In contrast to circulating fetal cells, fetal trophoblastic cells in maternal reproductive tract have been demonstrated to be a promising cell source for noninvasive genetic investigation. These fetal cells are released from the conceptus by an unknown mechanism and their number is much higher than that of circulating fetal cells (hundreds to thousands of fetal cells per specimen and about one fetal cell among 2000 maternal cells) (Figures S1 and S2, Supporting Information). However, although much progress has been made in understanding their cellular phenotype, there is still a lack of investigation on the molecular profiling of these cells. As trophoblastic cells are mixed with a large number of maternal epithelial cells, purification and identification processes are indispensable. Advances in isolation technology such as magnetic activated cell sorting based assays have allowed the separation of trophoblastic cells with little cell damage. However, traditional methods for the identification of such cells often require cumbersome steps of cell fixation and penetration. The intracellular labeling process may potentially affect the quality of DNA, which can influence the success of downstream molecular analysis. Detection of the cell-surface markers, such as HLA-G, seems to be a solution to address this problem. However, HLA-G is not specific enough and its expression is reduced in preeclamptic pregnancies. This may lead to low fetal cell purity. In this study, we demonstrate a novel cell-based noninvasive prenatal testing system, in which rare trophoblastic cells in Papanicolaou smears (Pap) can be nondestructively identified and isolated at single-cell level, thus providing pure fetal DNA with high integrity for fetal genotyping (Figure 1a). We showed that trophoblastic cells produced a higher fluorescence signal compared to maternal cells in endoplasmic reticulum (ER) staining. Trophoblastic cells could be clearly distinguished from abundant maternal cells by ER-based-LDA analysis (ER-LDA). Compared with intracellular labeling in vivo, ER-LDA has minimal effect on cell quality, thus ensuring the successful genotyping at single-cell level. Importantly, ER-LDA cell-based test showed high performance on the prenatal detection of thalassemia, one of the most common recessive monogenic diseases in southern China. This cell-based test may also be extended to the detection of other monogenic disorders.

2. Results

2.1. Establishment of ER-LDA Assay for Identifying Rare Trophoblastic Cells

To nondestructive identification of rare trophoblastic cells in Pap samples, a novel assay based on endoplasmic reticulum staining and linear discriminant analysis (ER-LDA) was developed. The continuous secretion of human chorionic gonadotropin (hCG) and a high level of progesterone are the hallmarks of trophoblastic cells. An important characteristic of these secretory cells is that their rough ER is particularly abundant, presenting an opportunity to distinguish fetal trophoblastic cells from maternal cells without immunolabelling. To validate this assumption, trophoblastic cells from trophoblastic cell lines, with squamous epithelial cells and white blood cells (WBCs), two major cell types in Pap specimens, were treated with ER-Tracker, a highly selective dye for ER staining. We found that the fluorescence intensity of ER-Tracker in trophoblastic cells was about 3.13 ± 1.13 (mean ± s.d.) and 2.64 ± 0.73 (mean ± s.d.) fold higher than that in squamous epithelial cells and WBCs, respectively (Figure 1b). In spiked-in samples (containing 500 trophoblastic cells, ≈200 000 squamous epithelial cells and WBCs), trophoblastic cells with a remarkable ER fluorescence could be easily identified by confocal imaging (Figure 1c).

Subsequently, we then used LDA analysis to improve the discrimination between trophoblastic and nontrophoblastic cells. The ER fluorescence and cell size of individual cells were used for LDA analysis. In the training cohort (trophoblastic cells = 1148, squamous epithelial cells = 258, WBCs = 948), the LDA showed a sensitivity of 96.51%, a specificity of 99.45% and an accuracy of 98.00% for discrimination of trophoblastic cells versus nontrophoblastic cells. Subsequently, a test cohort (trophoblastic cells = 200, squamous epithelial cells = 200, WBCs = 200) was used to demonstrate the capability of ER-LDA to identify trophoblastic cells among nontrophoblastic cells. An

---

**Figure 1.** Development of ER-LDA for analyzing rare trophoblastic cells. a) The working principle of ER-LDA cell-based test. ER^{high}/DAPI^{low} cells are analyzed by LDA, validated by STR, and isolated at single-cell level for sequencing. b) The relative fluorescence intensity of ER-tracker in three trophoblastic cell lines, HTR8/SVneo, JEG-3, and JAR, and in squamous epithelial cells and white blood cells taken from healthy female donors. The fluorescence intensity of each single cell is quantified by Image J software, and the relative fluorescence intensity between trophoblastic cells and squamous epithelial cells or white blood cells is calculated. Lines represent the mean values and the interquartile range of 3 replicates. c) ER^{high} trophoblastic cells imaged by confocal microscopy. The fluorescence intensity of each single cell is quantified by Image J software, and the relative fluorescence intensity between trophoblastic cells and squamous epithelial cells or white blood cells is calculated. Lines represent the mean values and the interquartile range of 3 replicates. d) The LDA predictive probability of 200 trophoblastic cells, 200 squamous epithelial cells and 200 leukocytes in test cohort. e) The coverage of WGA products assessed by the amplification of 22 genomic loci. f) Representative images of ER^{high} trophoblastic cells in 48 Pap samples. Cells are stained with ER-Tracker and DAPI. g) The LDA predictive probability of 209 candidate trophoblastic cells from 48 Pap samples. Two to 7 single cells per sample are analyzed and collected for WGA.
accuracy of 98.83% was obtained for identification of trophoblastic cells. Of the 198 cells predicted to be trophoblastic cells, the predictive probability of 193 (97.47%) cells was greater than 0.9 (Figure 1d). With the aid of ER-LDA, trophoblastic cells with a bright fluorescence could be isolated at single-cell level from spiked-in samples (Figure S3a, Supporting Information). These results indicate that the ER-LDA assay, with its high accuracy, is suitable for the rapid identification of fetal trophoblastic cells in Pap samples.

To test whether ER-LDA affects the quality of rare trophoblastic cells for molecular analysis, single JEG-3 cells identified by ER-LDA in spiked-in sample were isolated and used for whole genome amplification (WGA). WGA products were used as template to amplify 22 genetic loci across 22 chromosomes to assess the genomic coverage. We found that all the 22 genetic loci in five single cells showed successful amplifications, which supports their high genomic coverage (Figure 1e). In contrast, only 4–12 loci were detectable in single cells identified by immunofluorescence, indicating the low DNA quality (Figure 1e).

2.2. Isolation and Characterization of Rare Fetal Cells from Clinical Samples

We subsequently examined the feasibility of using ER-LDA in the investigation of clinical samples. Using this assay, a range of two to 19 putative fetal cells could be identified in 60 samples with gestational ages ranging from 4 to 38 weeks (Table S1, Supporting Information). As expected, putative trophoblastic cells showed a much brighter ER fluorescence (2- to 5-fold) than that of background cells (Figure 1f). In contrast, only 4–12 loci were detectable in single cells in 48 samples, 197 (94.26%) cells showed a probability from these samples showed positive probe binding for the Y chromosome, suggesting their fetal origin (Figure 2b).

2.3. Application in Thalassemia Detection

Thalassemia is one of the most common monogenic diseases, with a high prevalence in southern China. To investigate whether the rare fetal trophoblastic cells can be applied to noninvasive prenatal testing for thalassemia, 11 pregnancies whose fetuses were at risk for thalassemia were recruited (Table 1). In these cases, pregnant women or their spouses were found to carry pathogenic variants for thalassemia. The average gestational age of these pregnancies was 18.9 weeks (ranging 4–32 weeks). We first evaluated the feasibility of rare fetal trophoblastic cells for the detection of $\beta$-thalassemia, a common form of $\alpha$-thalassemia. The high-integrity of fetal DNA obtained from intact trophoblastic cells allows the detection of $\beta$-SEA/ $\alpha$-SEA thalassemia with only two simple steps: short fragment Gap-PCR and Sanger sequencing. Using short
Figure 2. Confirmation of the fetal origin of ER<sup>high</sup> trophoblastic cells. a) Representative immunostaining results of candidate cells isolated from sample P26. Twelve single trophoblastic cells are isolated, six cells are stained with β-HCG and six stained with HLA-G. NC, negative control, cells are not stained with antibodies. Scale bar, 15 µm. b) ER<sup>high</sup> trophoblastic cells isolated from seven pregnancies with a male fetus processed with FISH analysis. Representative results of single cells from P31, P35, and P37 are shown. Y chromosome signal is observed in these cells. Green fluorescence and red fluorescence, respectively, represent chromosome X and chromosome Y. c) The fragment size of the WGA product of P24 assessed by Agilent 2100 bioanalyzer. d) The STR profiles of maternal genomic DNA and fetal DNA amplified from rare fetal cells. 28 WGA samples with concentrations greater than 300 ng µL<sup>−1</sup> are used for STR analysis. The red squares represent the informative loci between fetal and maternal cells. e) SRY-PCR for detecting chromosome Y. SRY amplicons are detected in pregnancies with a male fetus. The actin beta (ACTB) gene is used as the internal reference.
fragment Gap-PCR, a fragment of 150 bp was amplified from the chromosome with the –SEA/ deletion and a fragment of 287 bp was amplified from the normal chromosome. Therefore, homozygotes for the –SEA/ deletion showed only a 150 bp fragment, carriers have both fragments and normal individuals have only the 287 bp fragment (Figure 3a). Trophoblastic cells from five cases whose fetuses were at risk for –SEA/ deletion were analyzed. For example, five and four trophoblastic cells were retrieved from subjects P5 and P30, respectively. For these subjects, a homozygous mutation, – SEA/–SEA in the parents carry the mutation of IVS-II-654(C>T) in the HBB gene associated with β-thalassemia (Figure 4d and Table 1). The genotype of the fetuses detected by trophoblastic cell-test in P2 and P50 was consistent with those of postnatal tests. In P54, we found normal allele in the fetus, which was consistent with the AC/CVS result, while the parents carry the mutation of IVS-II-654(C>T) in the HBB gene associated with β-thalassemia (Figure 4e and Table 1). Notably, in P59, paternal normal allele was identified in trophoblastic cells, while the mother had a molecular diagnosis of IVS-II-654(C>T) heterozygote (Figure 4f and Table 1).

### Table 1. Results of thalassemia related mutations in candidate fetal trophoblastic cells.

| Patient | Age | Gestational age (week) | Single cells | Genotypes for | Father | Mother | Fetus (real-cell analysis) | AC/CVS/ postnatal testing |
|---------|-----|------------------------|--------------|---------------|--------|--------|--------------------------|---------------------------|
| P1      | 29  | 22.1                   | 5            | α-globin      | –SEA/αα| –SEA/αα| –SEA/αα                  | –SEA/αα                   |
| P2      | 25  | 23.6                   | 6            | β-globin      | normal | CDs41/42(-TCTT) normal | CD41/42(-TCTT) normal    |
| P4      | 31  | 44                     | 6            | α-globin      | –SEA/αα| α/α   | –SEA/αα                  | –SEA/αα                   |
| P5      | 30  | 29.2                   | 5            | α-globin      | –SEA/αα| –SEA/αα| –SEA/αα                  | –SEA/αα                   |
| P18     | 19  | 13.4                   | 5            | α-globin      | α/α   | –SEA/αα| α/α                       | normal                    |
| P30     | 25  | 32.6                   | 4            | α-globin      | –SEA/αα| –SEA/αα| –SEA/αα                  | –SEA/αα                   |
| P50     | 17  | 10                     | 4            | β-globin      | normal | CD41/42(-TCTT) CD41/42(-TCTT) CD41/42(-TCTT) CD41/42(-TCTT) |
| P54     | 27  | 17.5                   | 5            | β-globin      | IVS-II-654(C>T) | normal | normal | normal | normal |
| P58     | 27  | 17.2                   | 4            | β-globin      | IVS-II-654(C>T) | –28(A>G) | –28(A>G) | –28(A>G) | –28(A>G) |
| P59     | 29  | 32                     | 4            | β-globin      | normal | IVS-II-654(C>T) normal | normal | normal | normal |
| P60     | 27  | 16                     | 7            | α and β-globin | –SEA/αα, –28(A>G) | normal | –28(A>G) | –28(A>G) |

2.4. Whole Exome Sequencing for the Detection of Other Monogenic Diseases

The high performance of trophoblastic cell-based test in thalassemia detection prompted us to test its feasibility in the detection of other monogenic diseases. We performed whole exome sequencing (WES) on rare fetal cells from five samples (Figure S8a, Supporting Information). Intact cells provided high-quantity amplified DNA for the successful library construction and sequencing. A mean of 143267 SNVs and 22407 InDels could...
Figure 3. Sequencing of candidate fetal cells from three pregnancies whose fetuses are at risk for \( -\text{SEA/} \) thalassemia. a) Short fragment Gap-PCR for detecting \( -\text{SEA/} \) mutation. A fragment of 150 bp is amplified from the chromosome with the \( -\text{SEA/} \) deletion and a fragment of 287 bp is amplified from the normal chromosome. b) For patient P30, the mother is heterozygous for \( -\text{SEA/} \) deletion, while the fetus is a homozygote. c) For patient P4, \( -\text{SEA/} \) heterozygous mutation is found in rare fetal cells but not found in maternal DNA. d) For patient P18, the mother is heterozygous for \( -\text{SEA/} \) deletion, while the fetus is normal. BF, bright field. Scale bar, 20 \( \mu m \). The white arrow indicates the single cells isolated from Pap samples and the black arrow indicates the breakpoint of the mutation. Fully symbols indicate affected members who carry homozygous mutation. Half-filled symbols indicate members who carry heterozygous mutation. Squares indicate males, circles indicate females, and diamonds indicate individuals of unknown gender.
be detected in these samples (Figure S8c, Supporting Information). A quality peak of 60 was observed for most variants in the genotype quality analysis and the average percentage of low quality variants (<60) was about 19.93%.[10] Importantly, the data on variants covered an average total number (HGMD: 763, Clinvar: 4047 and OMIM: 3307) of disease-associated genes across the whole genome, which allowed us to investigate the pathogenic mutations for diagnosing common monogenic diseases. For the 52 most common monogenic disease-associated genes, 77 variants in 39 genes were identified in these samples and plotted (Figure S8d, Supporting Information). These results demonstrated that the rare fetal cells showed good performance in detection of SNVs and the variants were considered of high quality. In addition, rare fetal cells could be successfully applied to analysis of CNVs (copy number variations), indicating their potential role in the detection of chromosome-related abnormalities (Figure S8b, Supporting Information). Overall, rare fetal cells may also be suitable for use in identifying inheritable mutations in a variety of monogenic diseases by high throughput sequencing.

3. Discussion

The technical advances in this work include nondestructive identification of rare trophoblastic cells for providing pure and high-quality of fetal DNA; characterization of the phenotype and confirmation of fetal origin of rare trophoblastic cells for accurate genotyping; a feasibility analysis of using the cell-based test for the detection of potential pathogenic mutations in the targeted genes of thalassemia. The presented antibody-free, simple, low-cost assay can be used to expand the current test for the detection of many more monogenic diseases, including recessive diseases and dominant diseases.

Figure 4. Sequencing of candidate fetal cells from six pregnancies whose fetuses are at risk for β-thalassemia. a) The sequencing results suggested that the fetus is normal for patient P2, while the mother harbors a CD514-42(CTTT) mutation for patient P50. b) The fetus and mother harbor a CD514-42(CTTT) mutation for patient P50. c) For patient P54, the father and the mother are heterozygous for IVS-III-654(C > T), while the fetus is normal. d) For patient P58, the mother is heterozygous for IVS-III-654(C > T), while the fetus is normal. e) For patient P59, the mother is heterozygous for IVS-III-654(C > T), while the fetus is normal. f) For patient P60, the mother is normal, while the fetus is heterozygous for IVS-III-654(C > T). The father harbors two variants, –28(A > G) in α-gene cluster and –28(A > G) in HBB gene. Scale bar, 20 µm. The white arrow indicates the single cells isolated from Pap samples and the black arrow indicates the location of the mutations. Fully symbols indicate affected members who carry homozygous mutation. Half-filled symbols indicate members who carry heterozygous mutation. Red symbols indicate individuals with the HBB mutations, and blue symbols indicate individuals with another mutation in the same family. Squares indicate males, circles indicate females, and diamonds indicate individuals of unknown gender. Single cells isolated from Pap samples are shown.
The collection of individual cells with high quality is critical for successful molecular analysis. However, the cumbersome and complicated processing required for intracellular labeling may result in a decline in the quality of target cells. Rapid screening of rare cells by exploiting the differences in biological activity of cellular organelles between target and background cells can be regarded as a sensitive choice.\(^{[26]}\) Trophoblasts with high ER activity represent an active secretory subset of trophoblastic cells in Pap samples from pregnant women.\(^{[27]}\) This distinctive feature enables the discrimination of rare fetal cells from maternal cells by simple steps of ER staining and LDA analysis. The enrichment-free and one-step labeling assay without cell fixation and penetration is considerably reduce damage to rare fetal cells. Importantly, using ER-LDA, we have achieved a high accuracy in distinguishing fetal trophoblastic cells from maternal cells. In real Pap samples, two to 19 candidate trophoblastic cells could be rapidly identified and the single cells we isolated from about 90.6% samples were validated as pure fetal cells. Furthermore, consistent with previous reports, trophoblastic cells could be found at an early gestational age of 4 weeks and are unaffected by maternal age.\(^{[14]}\) This nondestructive and highly accurate method is well suited for identifying single trophoblastic cells with high quality for molecular analysis.

Determining the fetal origin of putative cells is a prerequisite for using these cells in prenatal testing. We found that most of ER\(^{\text{high}}\) cells were β-HCG and HLA-G positive. Moreover, ER\(^{\text{high}}\) cells were also positive for Y chromosome in pregnancies with a male fetus in FISH analysis. All these findings suggest that most of ER\(^{\text{high}}\) cells were of fetal origin. In fact, it is not the phenotype, but the STR and SNP genotypes of putative cells that represent the gold standard for confirming their fetal origin. Currently, only one study has closely investigated the fetal origin of trophoblastic cells by sequencing a large panel of STRs and SNPs.\(^{[15]}\) The successful detection of STRs of rare trophoblastic cells in our study further supports the fact that ER-LDA is valid in obtaining pure fetal cells and that it is able to obtain fetal genetic information precisely by analyzing these rare fetal cells. With a high purity of fetal cells, the accuracy of fetal genotyping is comparable to that obtained by invasive strategies and may be superior to cfDNA.\(^{[28]}\)

Methods for prenatal diagnosis of many monogenic diseases with high incidence still rely on invasive procedures. Because of the limited amount and fragmented nature of fetal cfDNA, the cfDNA-based test has several limitations for detecting monogenic disorders.\(^{[29]}\) Few attempts have been made to investigate the role of cell-based test in the detection of monogenic disorders.\(^{[30,31]}\) Cell-based testing has several advantages over cfDNA-based test, in that fetal cells provide pure fetal DNA of high integrity.\(^{[32]}\) A total of 11 pregnancies whose fetuses may carry thalassemia mutations were investigated: five were at risk for \(\beta^{+}\)-thalassemia, and six were at risk for \(\beta\)-thalassemia. Thalassemia-related mutations could be reliably detected by analyzing these rare fetal cells. This is the first study to diagnose fetal thalassemia by using rare trophoblastic cells isolated from Pap samples. More work is ongoing to explore the possibility of utilizing this cell-based test for diagnosing other genetic diseases.

Some important limitations have to be considered in our study. Before our screening and diagnostic platform can be used in the clinic, further studies with a larger sample size are required and should include more patients with diverse genetic abnormalities. Owing to the limited length of the amplified DNA, this assay is presently not applicable for long fragment Gap-PCR for the detection of \(-\alpha^{+}\) and \(-\alpha^{0}\) thalassemia. Thus, a more powerful whole genome amplification method may be warranted to obtain a better quality of fetal DNA with an entire genome that is adequate for more molecular analyses.

Overall, the ER-LDA single cell platform presented here offers a noninvasive method for acquiring valuable molecular information from fetuses who are at risk for monogenic disorders. ER-LDA has high accuracy in identifying rare trophoblastic cells with high quality. This test based on rare cells has high diagnostic accuracy without safety risk for the fetus when compared with invasive procedures, giving it the potential to be used in the clinic. The findings of our study imply that detection of rare fetal cells in Pap samples by ER-LDA holds great promise for identifying fetuses with thalassemia, and other congenital defects.

### 4. Experimental Section

**Participants and Sampling:** From October 2017 through May 2019, a total of 232 Pap samples, including 167 from singleton pregnancies and 65 from nonpregnant controls, were collected from Nanfang Hospital, Southern Medical University (Guangzhou, China). Clinical information, including maternal age, gestational week and genetic testing results, was also collected. All samples were confirmed to be free of sperm contamination under a microscope. Samples with sperm contamination or with damaged cells were excluded from the study. This study was approved by the Ethics Committee of Nanfang Hospital, Southern Medical University and prior informed consent was provided by all participants. Specimens were safely obtained using a cotton swab, as described previously.\(^{[32]}\) Briefly, the cotton swab was rotated 360° at the external os, as the routine of Pap sampling, and stored in 5 mL of liquid-based cytology test (LCT) fixative solution (Hologic, Inc., Marlborough, MA). Samples were stored at 4 °C, transported to the laboratory using freezer packs and processed within 5 h. Cells were harvested by centrifugation at 4000 rpm for 5 min and the cell pellet was washed with phosphate buffered saline (PBS) twice. Following this, the cell pellet was re-suspended in 2 mL of PBS for ER staining and single-cell isolation immediately. The samples used for PCR analysis were stored at −80 °C and processed within 2 months of collection.

**Cell Lines:** Three human trophoblastic cell lines (HTR8/SVneo, JEG-3 and JAR) were purchased from the American Type Culture Collection. HTR8/SVneo and JAR cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Inc.) and JEG-3 cells in Dulbecco’s modified Eagle medium (DMEM, Gibco, Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, Inc.). Both cell lines were cultured in an atmosphere of 5% CO\(_2\) at 37 °C. Trophoblastic cells were collected for spiked-in experiments and LDA analysis.

**DNA Extraction and Quantitative PCR:** To assess the presence and approximate number of trophoblastic cells in Pap samples, the copy number of the SRY (sex determining region of chromosome Y) gene, representing the number of fetal cells in samples from pregnancies with a male fetus, was calculated by ddPCR and qPCR (Figure S1, Supporting Information). DNA was extracted from the cell pellets of 172 Pap specimens (107 from pregnancies and 65 from nonpregnant women) using the commercial TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). The Y chromosome copy number was determined by Droplet Digital PCR (ddPCR, Bio-Rad QX200, Hercules, CA, USA) and Quantitative PCR (qPCR, Roche Cobas Z480, Basel, Switzerland). For ddPCR, the 20 μL ddPCR mixture consisted of 10 μL of 2 × ddPCR EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 500 × 10⁻⁹ μ of
SRY primers (Table S3, Supporting Information), and 100 ng of DNA template. The mixtures were transferred to sample wells of a droplet generator cartridge (BioRad, Hercules, CA, USA); 70 µL of Droplet Generation Oil was loaded into the oil wells, and ~20,000 water-in-oil droplets were generated in each outlet well. After that, the droplets were transferred to a 96-well plate and amplified with the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and annealing temperature (Table S3, Supporting Information) for 30 s and 1 cycle of 72 °C for 5 min. After PCR, the 96-well plate was loaded on the QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA) and the data were acquired using QuantaSoft analysis software (Bio-Rad, Hercules, CA, USA). For the SRY qPCR, the 20 µL reaction mixture consisted of 1 × SYBR Green master mix (Takara Bio, Da Lian, China), 500 × 10⁻⁹ M of primers, and 100 ng of DNA template. The PCR conditions for the qPCR were the same as those for the ddPCR.

**Screening of Candidate Cells:** Aliquots of 2 mL cell suspension from Pap samples were first filtered by a 40 µm cell strainer (MACS Smart Strainer) to remove large epithelial cells. The cell pellet was washed with PBS and incubated with 1 × 10⁻⁹ M ER-Tracker Red (Keygen Biotech, Jiang Su, China) for 30 min and then stained with 300 × 10⁻⁹ M DAPI (Invitrogen, MA, USA) for 15 min. Cells were centrifuged and washed with PBS, and 70 µL of cell suspension was applied to a slide and left for 3 min for the cells to settle. Each slide was scanned and imaged using an EVOS FL automated Cell Imaging System (Thermo Fisher Scientific, Massachusetts, USA) in two fluorescent colors (ER-Tracker: Red, DAPI: Blue) and the bright field. Cells were screened at low power (×10) magnification and identified at high power (×40) magnification. The ER-Tracker fluorescence intensity and cell size of candidate cells were measured using Cellette Image Analysis Software.

**Linear Discrimination Analysis (LDA):** LDA was used to discriminate trophoblastic cells from maternal cells. Using the ER-Tracker fluorescence intensity and cell size signature of each cell as input, the LDA was implemented in SPSS version 19.0 (IBM Inc., Chicago, IL, USA). Trophoblastic cells from cell lines, squamous epithelial cells and leukocytes from healthy female individuals were used as the training cohort and the test cohort. The LDA algorithm for discriminating trophoblastic cells from epithelial cells and leukocytes was determined. Subsequently, this LDA algorithm was applied to the clinical sample cohort. The probability of each candidate cell to be predicted as a trophoblast cell was obtained.

**Whole Genome Amplification:** A total of 48 samples were processed and characterized immediately after collection from patients. ER⁺/− cells in these samples were used for WGA. Candidate cells on the slides were individually picked using glass micropipettes (40 µm in diameter) and confirmed on a new slide. Single isolated cells were then transferred into PCR tubes containing 4 µL of PBS solution. These cells were amplified using a REPLi-g Single Cell Kit (Qiagen, Hilden, Germany) according to the instructions. Briefly, PCR tubes containing single cells were centrifuged for 2 min, and incubated with 3 µL of denaturation buffer at 65 °C for 10 min. After that, 3 µL of stop solution and 40 µL of master mix were added, respectively. Subsequently, the tubes were incubated at 30 °C for 8 h for amplification.

**Quality Assessment of WGA Products:** First, the size distribution of the WGA products was evaluated using the Agilent 2100 bioanalyzer system (Agilent Technologies, Waldbronn, Germany). The WGA products were loaded on DNA 1000 Lab Chips according to the manufacturer’s instructions. Briefly, 9 µL of gel-dye mixture was loaded on the micro-channels using a 1 mL syringe; 6 µL of ladder mixture or WGA samples were loaded on the ladder well and sample wells, respectively. Chips were mixed using a Vortex Mixer at 2400 g for 1 min and immediately processed by the bioanalyzer. The results were analyzed using Agilent 2100 Expert Software (Agilent Technologies, Waldbronn, Germany).

**STRA Analysis:** For STR genotyping, WGA products from rare trophoblastic cells and maternal gDNA were investigated using a PowerPlex System according to the manufacturer’s protocol, and 19 human STR loci were detected: D1S1656, D2S1338, D3S1358, D5S818, D6S1043, D7S820, D8S1179, D12S391, D13S317, D16S539, D18S511, D19S433, D21S11, CSF1PO, Penta D, FGA, TH01, vWA, and Penta E. The PCR fragments were analyzed using an ABI3130xl Genetic Analyzer (Applied Biosystems Inc., Massachusetts, USA) and alleles were identified using GeneMapper 4.2 (Applied Biosystems Inc., Thermo Fisher Scientific, Massachusetts, USA).

**Fluorescence Activated Cell Sorting:** For fluorescence activated cell sorting (FACS), cells from Pap samples were incubated with 2 µg mL⁻¹ of FITC-labeled anti-HLA-G (MEM-G/11, Invitrogen, MA, USA) at room temperature (RT) for 3 h in the dark. Cells were washed with PBS twice and processed on a MoFlo XDP Cell Sorter (Beckman Coulter Inc., CA, USA). The data were analyzed using Summit 5.2 software and the separated HLA-G positive cells were used for whole genome amplification.

**Screening of Fetal Cells from Thalassemia Samples:** Trophoblastic cells that were ER⁺/−/DAPI⁺ were retrieved and amplified, as described above, from pregnancies with a fetal risk of thalassemia. The WGA product was then purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany), and its concentration was determined by Qubit 3.0 (Invitrogen, Massachusetts, USA). Amplification of target regions was conducted using the primers and the PCR conditions listed in Table S3 (Supporting Information). The purified PCR product was analyzed with Sanger sequencing (Applied Biosystems Inc., Massachusetts, USA).

**Whole Exome Sequencing:** For each sample, 1 µg of WGA product was randomly fragmented by Covaris (Woburn, MA, USA). Fragmented DNA with an average size of 200–400 bp was selected using an Agencourt AMPure XP-Medium kit (Beckman Coulter, NSW, Australia). After the processes of end-repair, 3’ adenylation, adapter-ligation and PCR amplifying for the selected fragments, the PCR products were purified using an Agencourt Mag PCR Clean Up Kit (Aygen, CA, USA). The purified products were used for hybridization with BGI hybridization kits. After that, the hybridization products were recovered using the Agenprep Mag PCR Clean Up Kit. The PCR products were heat denatured and single-stranded DNA was circularized by the splint oligo sequence. The single strand circle DNA (ssCir DNA) was used as the final library and qualified by QC. The circular DNA was then amplified with Phi29 DNA polymerase. The formed DNA nanoballs (DNB) were loaded on a patterned nanoarray. Finally, the pair-end 100-base reads were generated by sequencing combinatorial Probe-Anchor Synthesis (cPAS) on BGISEQ-1000.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Y.H., B.S., and L.H. contributed equally to this work. The authors thank Department of Obstetrics and Gynecology of Nanfang Hospital for collecting samples for this study. The authors thank International Science Editing for editing this manuscript. This study was supported by the National Natural Science Foundation of China (81871735) and the Outstanding Youths Development Scheme of Nanfang Hospital, Southern Medical University (2018002), and the Outstanding Youths Development Scheme of Southern Medical University (2019YQPY007).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

monogenic diseases, nondestructive identification, noninvasive prenatal testing, single-cell isolation, trophoblastic cells

Received: November 24, 2019
Revised: January 27, 2020
Published online: February 13, 2020

[1] R. J. Wapner, Semin. Perinatol. 2005, 29, 401.
[2] F. Mujerievovic, Z. Alrefiea, Obstet. Gynecol. 2007, 110, 687.
[3] L. S. Chitty, D. W. Bianchi, Prenat Diagn. 2013, 33, 511.
[4] S. Dan, W. Wang, J. Ren, Y. Li, H. Hu, Z. Xu, T. K. Lau, J. Xie, W. Zhao, H. Huang, L. Sun, X. Zhang, S. Liao, R. Qiang, J. Cao, Q. Zhang, Y. Zhou, H. Zhu, M. Zhong, Y. Guo, L. Lin, Z. Gao, H. Yao, H. Zhang, L. Zhao, F. Jiang, F. Chen, H. Jiang, S. Li, J. Wang, T. Duan, Y. Su, Prenat Diagn. 2012, 32, 1225.
[5] C. Vermeulen, G. Geeven, E. de Wit, M. Verstegen, R. P. M. Jansen, M. van Kranenburg, E. de Bruijn, S. L. Pult, E. Kruisselbrink, Z. Shahsavari, D. Omrani, F. Zeinali, H. Najmabadi, T. Katsila, C. Vrettou, G. P. Patrinos, J. Traeger-Synodinos, E. Splinter, J. M. Beekman, S. Kheradmand Kia, G. J. Te Meerman, H. K. Plos van Ameestal, W. de Laat, Am. J. Hum. Genet. 2017, 101, 326.
[6] J. A. Canick, G. E. Palomaki, E. M. Kloza, G. M. Lambert-Messerlian, J. E. Haddow, Prenat Diagn. 2013, 33, 667.
[7] K. C. Chan, J. Zhang, A. B. Hui, N. Wong, T. K. Lau, T. N. Leung, K. W. Lo, D. W. Huang, Y. M. Lo, Clin. Chem. 2004, 50, 88.
[8] A. L. Beaudet, Am. J. Med. Genet., Part C 2016, 172, 123.
[9] M. Fiddler, J. Clin. Med. 2014, 3, 972.
[10] F. Chen, P. Liu, Y. Gu, Z. Zhu, A. Nannisetti, Z. Lan, Z. Huang, J. S. Liu, X. Kang, Y. Deng, L. Luo, D. Jiang, Y. Qiu, J. Pan, J. Xia, X. Xiong, C. Liu, L. Xie, Q. Shi, J. Li, X. Zhang, W. Wang, S. Drnanac, L. Bolund, H. Jiang, R. Drnanac, X. Xu, Prenat Diagn. 2017, 37, 1311.
[11] S. Kolovraa, R. Singh, E. A. Normand, S. Qdaisat, I. B. van den Veyer, L. Jackson, L. Hatt, P. Schelde, N. Uldbjerg, E. M. Vestergaard, L. Zhao, R. Chen, C. A. Shaw, A. M. Breman, A. L. Beaudet, Prenat Diagn. 2016, 36, 1127.
[12] S. Hou, J. F. Chen, M. Song, Y. Zhu, Y. J. Jan, S. H. Chen, T. H. Weng, D. A. Ling, S. F. Chen, T. Ro, A. J. Liang, T. Lee, H. Jin, M. Li, L. Liu, Y. S. Hsiao, P. Chen, H. H. Yu, M. S. Tsai, M. D. Pisarska, A. Chen, L. C. Chen, H. R. Tseng, ACS Nano 2017, 11, 8167.
[13] A. Emad, E. F. Bouchard, J. Lamoreux, A. Ouellet, A. Dutta, U. Klingbeil, R. Drouin, Prenat Diagn. 2014, 34, 538.
[14] J. M. Bolnick, B. A. Kilburn, S. Bajpayee, N. Reddy, R. Jeliani, B. Crone, N. Simmerman, M. Singh, M. P. Diamond, D. R. Arman, Fertil. Steril. 2014, 102, 135.
[15] C. V Jain, L. Kadam, M. van Dijk, H. R. Kohan-Ghadir, B. A. Kilburn, C. Hartman, V. Mazzorana, A. Visser, M. Hertz, A. D. Bolnick, R. Fritz, D. R. Arman, S. Drewlo, Sci. Transl. Med. 2016, 8, 363rr4.
[16] M. Adinolfi, J. Sherlock, Hum. Reprod. Update 1997, 3, 383.
[17] A. N. Imudia, Y. Suzuki, B. A. Kilburn, F. D. Yelian, M. P. Diamond, R. Romero, D. R. Arman, Hum. Reprod. 2009, 24, 2086.
[18] R. Cheng, F. Zhang, M. Li, X. Wo, Y. W. Su, W. Wang, Front. Chem. 2019, 7, 588.
[19] R. Mitra, O. Chao, Y. Urasaki, O. B. Goodman, T. T. Le, BMC Cancer 2012, 12, 540.
[20] S. M. Yie, L. H. Li, Y. M. Li, C. Librach, Am. J. Obstet. Gynecol. 2004, 191, 525.
[21] X. Zhu, T. Han, G. Yin, X. Wang, Y. Yao, Hypertens. Pregnancy 2012, 37, 252.
[22] K. Lai, G. Huang, L. Su, Y. He, Sci. Rep. 2017, 7, 920.
[23] R. E. Canfield, F. J. Morgan, S. Kammerman, J. J. Bell, G. M. Agosto, Recent Prog. Horm. Res. 1971, 27, 121.
[24] A. M. Benham, Cold Spring Harbor Perspect. Biol. 2012, 4, a012872.
[25] T. M. Ko, L. H. Tseng, F. J. Hsieh, P. M. Hsu, T. Y. Lee, Hum. Genet. 1992, 88, 245.
[26] Y. Tang, Z. Wang, Z. Li, J. Kim, Y. Deng, Y. Li, J. R. Heath, W. Wei, S. Lu, Q. Shi, Proc. Natl. Acad. Sci. USA 2017, 114, 2544.
[27] D. M. Nelson, R. K. Meister, J. Ortman-Nabi, S. Sparks, V. C. Stevens, Placenta 1986, 7, 1.
[28] J. Zhang, L. Li, J. B. Sauzier, Y. Feng, Y. Jiang, J. Simson, A. K. McCombs, E. S. Schmitt, S. Peacock, S. Chen, H. Hai, X. Ge, G. Wang, C. A. Shaw, H. Mei, A. Breman, F. Xia, Y. Yang, A. Purgason, A. Pourpak, Z. Chen, X. Wang, Y. Wang, S. Kulikarni, K. W. Choy, R. J. Wapner, I. B. Van den Veyer, A. Beaudet, S. Parmar, L. J. Wong, C. M. Eng, Nat. Med. 2019, 25, 439.
[29] Y. M. Lo, K. C. Chan, H. Sun, E. Z. Chen, P. Jiang, F. M. Lun, Y. W. Zheng, T. Y. Leung, T. K. Lau, C. R. Cantor, R. W. Chiu, Sci. Transl. Med. 2010, 2, 61ra91.
[30] I. Pfeifer, A. Benachi, A. Saker, J. P. Bonnefont, H. Mouawia, L. Broncy, R. Frydman, M. L. Brival, B. Lacour, R. Dachez, P. Paterlini-Brechot, Reprod. BioMed. Online 2012, 25, 508.
[31] L. Vossaat, Q. Wang, R. Salmon, X. Zhuo, C. Qu, D. Henke, R. Seubert, J. Chow, L. O’Reen, B. Enright, J. Stilwell, E. Kaldjian, Y. Yang, C. Shaw, B. Levy, R. Wapner, A. Breman, I. Van den Veyer, A. Beaudet, Prenat Diagn. 2018, 38, 1069.