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University Honors Thesis

Prenatal Genetic Diagnosis Using Transcervically Derived and Immunomagnetically Isolated Trophoblast Cells

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ABSTRACT

Trophoblast cells migrate from the placenta into the endocervical canal early in the first trimester and can be collected non-invasively by transcervical sampling (TCS), potentially providing fetal DNA for prenatal genetic diagnosis. Experiments were conducted to separate fetal cells from maternal cells within the TCS specimens and use the fetal cells to perform genetic analysis. Trophoblast cells were efficiently isolated from TCS specimens during weeks 5-18 of gestation using HLA-G antibody coupled to magnetic nanoparticles. Immunofluorescence microscopy revealed that 99% of isolated cells expressed the chorionic gonadotropin β subunit (β-CG), while the non-bound cell fraction expressed none. Immunomagnetically isolated cells from 20 patient specimens

were used to determine fetal gender in single cell assays, amplifying sequences in genes on the X (*DMD*) and Y (*SRY*) chromosomes by multiplex polymerase chain reaction (PCR). Of 270 total cells, PCR products were detected in 256 cells and identified gender (11 male; 9 female) without error. PCR-based genetic testing is highly reliable as early as week 5 of gestation after immunomagnetic isolation of extravillous trophoblast cells obtained by TCS.

INTRODUCTION

Prenatal genetic diagnosis is an appropriate course of action to take in a pregnancy when it is known that one of the biological parents has a genetically inheritable disease or when the biological mother's age is so advanced that it may introduce genetic abnormalities to the embryo. In fact, there is a 3% to 5% chance that an infant will be born with a chromosomal abnormality or a structural defect (O'Leary et al., 2006). Yet, as of now, there are many limitations as to how genetic testing can take place. The earliest that genetic testing can be done is in the first trimester after 10 to 12 weeks of pregnancy using chorionic villus sampling (CVS). It is not the most reliable method, and it puts the woman at risk for infection or a 2% chance of miscarriage (Mujezinovic and Alfirevic, 2007). There is also evidence that CVS can cause preeclampsia, a condition some pregnant women develop in which their blood pressure increases and excess protein is found in their urine (Silver et al., 2005). Preeclampsia can be fatal to both the mother and the baby, and it typically necessitates an early delivery with

prematurity. An alternative method to CVS is amniocentesis. Amniocentesis can only be performed in the second trimester between the woman's 15th and 20th week of pregnancy. It is also an invasive method that puts the pregnancy at a 1.9% chance of miscarriage (Mujezinovic and Alfirevic, 2007). Operator experience is a major factor in safety (Wapner, 2005), and the risk of pregnancy loss is usually less than 1% with an experienced physician.

Through this research project, an alternative method for prenatal genetic diagnosis was explored. This method is based on a non-invasive method that can be performed earlier on in the pregnancy (Imudia et al., 2009). Cervical specimens similar to those collected for a PAP smear contain mostly cells shed from the endocervical canal. However, during weeks 6-14 of pregnancy, trophoblast cells migrating from the placenta reside in the cervix and can be identified by their expression of embryo-specific proteins, including HLAG (Imudia et al., 2010). Time was taken to develop an isolation technique for separating HLAG positive cells from HLAG negative cells using a trophoblast cell line capable of HLAG expression. The method developed uses specific antibodies bound to magnetic nanoparticles to isolate the trophoblast cells, which have now been successfully isolated them from actual cervical specimens for genetic testing of the fetus. This provides the mother with an earlier time frame to decide how to handle her pregnancy if an embryonic genetic or chromosomal abnormality does show up. It will also eliminate the risk of miscarriage, as it is a non-invasive technique.

These experiments were proposed to demonstrate proof-of-principle that isolation of trophoblast cells is indeed possible when combined with an excess of different cells to simulate a cervical sample and that single-cell methodology can be used to assess the purity and fetal origin of isolated trophoblast cells by DNA fingerprinting.

Initially, a human trophoblast cell line (HTR) was used to isolate cells expressing an embryo-specific protein from a solution containing an excess of cells that lack the protein. Once this method was developed, multiple cervical samples collected from clinics that our lab collaborates with were used to demonstrate that the method is feasible using actual cervical samples. Once the cervical samples were collected from patients in the clinics, the samples were fixed in the lab using Methanol Acetic Acid to prevent the cells from changing between the time samples were received and the time that they were tested. After the cells were fixed, they were separated based on their expression of HLAG. The cells were incubated with magnetic beads that have HLAG antibody coupled to their surface. Thus, all of the HLAG positive cells became bound to the magnetic beads, and the HLAG negative cells remained free of beads. The cell mixture was then magnetized, the idea being that all of the bead-bound cells should be attracted towards the magnet. Discarding the supernatant and sufficiently washing the cells bound to the magnet obtained a pure sample of HLAG positive cells. This method was tested using cultured trophoblast cells that were forced to

express HLAG. The method was tested again by immunofluorescence labeling with antibody against beta-HCG using cells that were recovered by immunomagnetic separation. Only human trophoblast cells express beta-hCG and should have bind the antibody. This experiment allowed the establishment of whether or not embryonic cells could be separated from maternal cells within an actual cervical sample and determined the purity and recovery (both in terms of percentage) of the HLAG positive cells when compared to the expected ratio. In preliminary studies, cervical samples collected from pregnant patients between weeks 7-10 were analyzed by beta-hCG immunofluorescence. A majority of trophoblast cells were recovered, and beta-hCG testing was 100% positive (as shown by the table below) proving that the cells recovered were indeed uncontaminated and HLAG positive.

Table 1. Trophoblast isolation from cervical specimens. Two specimens collected from pregnant patients between Weeks 7-10 were analyzed by immunohistochemistry to determine trophoblast frequency (HLA-G+ :Total cells) and predict the number of trophoblast cells expected from the total number used. Approximately 10% of the specimens were processed by immunomagnetic affinity isolation to purify trophoblast cells. Isolated cells were counted for comparison with the number of trophoblast cells expected. Aliquots of the separated Bound cells and Non-bound cells from Samples# 8.4 and 8.25 were labeled with anti-β-hCG to determine the percentage of cells expressing the trophoblast-specific protein in each group.

Sample ID	**Trophoblast Frequency**	**No. Cells Processed**	**No. Cells Expected**	**No. Cells Isolated**	**Trophoblast Recovery**	**β-hCG + Bound Cells**	**β-hCG + Non-Bound Cells**
8.4	1:1446	500,000	346	365	105%	100%	0%
8.25	1:1860	240,000	129	78	60%	100%	0%

MATERIALS AND METHODS

Separating cells based on an embryo-specific marker.

A human trophoblast cell line (HTR) was used to demonstrate that it is possible to isolate cells expressing an embryo-specific protein from a solution containing an excess of cells lacking that protein. The HTR cell line was cultured using two different methods. The HTR cell line was cultured on fibronectin and matrigel, respectively, both basement membranes. Fibronectin does not affect the HTR cells, while Matrigel forces them to express HLAG (human leukocyte antigen G), which is only expressed by embryonic cells. Cervical specimens collected from pregnant women during the first trimester contain both maternal (cervical) and embryonic (trophoblast) cells. Thus, a mock cervical specimen was created as represented by the HLAG positive and HLAG negative trophoblast cells. After the cells were cultured, the cells were fixed and permeabilized. The HLAG positive cells were then stained with propidium iodide, a fluorescent red nuclear stain. The HLAG negative cells were stained with DAPI, a fluorescent blue nuclear stain. The cells were then be counted and mixed together in a 1:2000 HLAG positive to negative ratio. This ratio is consistent with the ratio of embryonic cells to maternal cells that are collected from cervical samples (Imudia et al., 2009). Once the cells were combined, they were separated based on their expression of HLAG. The mixture of cells was incubated with magnetic beads that had HLAG antibody coupled to their surface. Thus, all of the HLAG positive cells were bound to the magnetic beads, and the HLAG negative cells remained

free of beads. The cell mixture was then magnetized, the idea being that all of the bead-bound cells should be attracted towards the magnet. By discarding the supernatant and sufficiently washing the cells bound to the magnet, it became possible to obtain a pure sample of HLAG positive cells. The success of this separation was determined by fluorescence microscopy, which identified cells labeled with propidium iodide or DAPI. If only a red fluorescent stain was visible, that meant the HLAG positive cells were pure. A blue fluorescent stain should not have been visible, as it would indicate that the isolated cells were impure. After undergoing this experiment, it was determined that the separation was indeed pure. This experiment established that embryonic cells could be separated from maternal cells based on HLAG expression. The purity and recovery (both in terms of percentage) of the HLAG positive cells was also determined by this experiment. In preliminary studies, this approach was used with a total of 100,000 cells containing 50 HLAG positive cells, and was able to recover approximately 10-20% of the HLAG positive cells free of contaminating HLAG negative cells. Recovery was not increased, and it was determined at a later point that this was because cells induced to express HLAG slowly and steadily lost their HLAG expression. When the experiment was repeated using trophoblast cells derived from transcervical specimens, recovery peaked at 95-100%.

X and Y chromosome assessment of isolated cells and preliminary experiments using HTR and fibroblast cells to refine method of PCR and storage.

Since the method being developed revolved around using a single-cell approach to conduct genetic testing, single-cell PCR was the first technique to be refined. Cells were diluted down until very low concentrations of cells or single cells were present in small volumes of RNase free water. Cells were then heated to boiling point in a PCR tube with RNase free water using a heating block, which lysed the cells to release the genomic DNA. Immunomagnetically isolated cells from these samples were used to conduct single cell assays by amplifying sequences in genes on the X (*DMD*) and Y (*SRY*) chromosomes by multiplex polymerase chain reaction (PCR). Preliminary steps have been taken and correct amplified products can be seen from single-cell PCR. Single bands were visible at the site of the X amplicon for HTR cells, which are female. The fibroblast cell line, derived from human foreskin, generated two bands representing both X and Y amplicons. This experiment ensured accurate results through PCR. Thus, when immunomagnetically isolated cells from 20 patient specimens were used to determine fetal gender in single cell assays, in the 270 total cells, PCR products were detected in 256 (95%) and identified gender (11 male; 9 female) without error. It was also vital to determine how the cervical samples would be stored before PCR is conducted. Time trials with single cells from the cell lines were conducted to see if PCR needed to be conducted immediately after cervical samples are received or whether the cells from the samples could be isolated

and stored frozen before conducting PCR. Based on the PCR results, it was determined that storage time did not affect results drastically. Thus, cervical samples were allowed to be frozen before undergoing PCR.

Genetic assessment of DNA using a single-cell approach.

Genetic assessment of DNA was conducted using the HTR and fetal fibroblast cell lines. Once the genetic assessment of DNA was completed using these mock cells, the refined procedure was to work with transcervically derived trophoblast cells. Because the maternal and embryonic cells in a cervical sample cannot be differentially labeled before the cell separation procedure, the isolation of pure trophoblast cells was further assessed (after completing beta-hCG testing) by DNA fingerprinting. Preliminary experiments were carried out to optimize DNA analysis protocols before proceeding with pilot cell separation experiment using HLAG positive HTR cells and a genetically distinct HLAG negative cell line. Cells in small groups or single cells were lysed for genomic DNA isolation and amplification through heating, as there is no commercial kit available at the time being that works with such low numbers of cells (similar to what was done with single-cell PCR). The cells were placed in RNase free water then boiled in order to lyse the cells and release the genetic content. Cells were lysed for genomic DNA isolation and amplification with the Whole Genome Amplification (WGA) Kit (Sigma Aldrich, St. Louis, MO) or Rubicon PicoPlexTM WGA (Rubican, Ann Arbor, MI), calculating yields by Nanodrop spectrophotometry (Wilmington, DE). DNA was isolated from each cell line to

establish their DNA fingerprints. Next, the whole genome amplification procedure was piloted with decreasing numbers of cells, down to single cells, followed by DNA fingerprinting. DNA was then fingerprinted using the AmpFlSTR Identifiler PCR Amplification kit (ABI) that was developed in accordance with forensic standards (Collins et al., 2004). As two different cell lines were used initially, the DNA fingerprints should have been different. If the fingerprints were consistent and differed between cell lines, the testing could be progressed from cell lines to transcervical samples. In this experiment reference fingerprints needed to be obtained that would serve as a model for distinguishing DNA from the cells. It was also important to acquire the ability to perform this procedure with the small number of cells isolated in the cell separation procedure used with cervical specimens. Despite diligent efforts, this method has not yet become feasible for a single-cell approach. The method was however successful on as little as 40 cells, which is substantially lower than the average of range of thousands of cells that are normally required for this procedure.

RESULTS

Twenty-six transcervical specimens were obtained from pregnant women with fetuses ranging in gestational age between 5 and 18 weeks (Table 2). Cell counts suggested that trophoblast recovery after immunomagnetic isolation averaged 101%. The average percentage of cells expressing β-CG among the magnetically bound cells was 99% (range, 95%-100%; Table 2), while none of the cells in the excluded, non-bound fraction were β-CG positive. To further

establish that the isolated cells were trophoblast, immunofluorescence microscopy was used to examine expression of marker proteins, β-CG, as expressed by immunomagnetically isolated cells from transcervical specimens.

Table 2. Trophoblast Content, Recovery and Purity. TCS specimens obtained from patients at the indicated gestational ages were examined by IHC for the presence of HLA-G and the total number of HLA-G-positive cells in each specimen was estimated. Anti-HLA-G-binding cells were isolated with magnetic nanoparticles and the total number of cells recovered from each specimen was estimated from cell counts, with the percentage of that predicted by IHC shown in parentheses. Immunofluorescence microscopy was used to determine the number of cells expressing β-CG/total cells examined, with the percentage expressing β-CG shown in parentheses.

Gestational Age (weeks.days)	# HLA-G-Positive Cells	# Isolated Cells (% Recovered)	β-CG Reactive (%)
5.3	1313	1140 (87)	233/233 (100)
6	609	998 (164)	190/200 (95)
7	575	593 (103)	118/118 (100)
7.3	677	855 (126)	540/542 (99.6)
7.4	842	623 (74)	100/102 (98)
7.5	314	870 (277)	250/250 (100)
7.6	1108	345 (31)	1482/1500(98.8)
8	593	510 (86)	250//250 (100)
8	1045	728 (70)	93/94 (98.9)
8	609	660 (108)	58/58 (100)
8	788	848 (108)	69/70 (99)
9.2	1260	248 (20)	1485/1500 (99)
10	2222	1462 (66)	793/811 (97.8)
10	1095	1020 (93)	84/84 (100)
11	581	720 (124)	594/598 (99.3)
11	1027	622 (61)	250/250 (100)
11	529	1463 (277)	181/191 (98.4)
12	850	578 (68)	247/250 (98.9)
12	879	758 (86)	96/96 (100)
12.4	1109	495 (45)	53/55 (96.4)
14	820	705 (86)	87/87 (100)

	14.6	466	660 (142)	1485/1500 (99)
	15.2	570	570 (100)	385/387 (99.5)
	15.4	2467	832 (34)	992/1000 (99.2)
	17.5	1009	818 (81)	850/853 (99.6)
	17.6	239	270 (113)	966/970 (99.6)
Average	**10.4**	**908**	**746 (101)**	**99%**

To establish that trophoblast cells isolated from transcervical specimens were a valid source of fetal DNA for single cell prenatal genetic diagnosis, multiplex PCR was conducted with replicates of individual frozen cells from 20 patient specimen 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, while trophoblast cells from female fetuses produced only the X band. In addition to individual trophoblast cells, PCR analysis of each specimen included single foreskin fibroblasts or isolated foreskin DNA as positive male controls. The fetal sex of all 20 pregnancies was correctly determined, as verified by delivery records (Table 3). There were nine female and eleven male fetuses among the twenty specimens examined. The specificity of the PCR worked well, as *SRY* amplicons were clearly distinguished from *DMD* amplicons. Overall, there were no visible bands produced by PCR in 14 of 270 cells evaluated (5.2%). The PCR-determined genders of individual cells were homogeneous within every specimen.

Table 3. Fetal Gender Determination. Anti-HLA-G-bound cells isolated with magnetic nanoparticles 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. Genders were verified from birth records.

Gestational Age (weeks.days)	X Chromosome detected (%)	Y Chromosome detected (%)	Gender Verification
5.3	10/10 (100)	0/10 (0)	Female
6	25/30 (83.3)	0/30 (0)	Female
7.6	25/25 (100)	0/25 (0)	Female
8	10/10 (100)	0/10 (0)	Female
10	10/10 (100)	0/10 (0)	Female
12	10/10 (100)	0/10 (0)	Female
12	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	25/28 (89.3)	25/28 (89.3)	Male
9.2	10/10 (100)	10/10 (100)	Male
10	10/10 (100)	10/10 (100)	Male
11	10/10 (100)	10/10 (100)	Male
12	24/26 (92.3)	24/26 (92.3)	Male
14	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

FUTURE DIRECTIONS

This method was implemented in all experiments, especially when cervical cell samples from pregnant patients obtained by physicians in the Department of

Obstetrics & Gynecology were used. If fingerprinting can be refined for use with a single-cell, then it will be establish whether the isolated cells are free of maternal DNA, which can easily be determined with a blood sample from the mother. It should be noted, however, that purity of the fetal cells may eventually become less important than yield. New technologies are currently being developed that use bioinformatics to distinguish maternal from fetal sequence information (Chiu et al., 2008; Jorgez and Bischoff, 2009; Lo et al., 2007; Lun et al., 2008).

By completing this study with the use of cervical cell samples from pregnant patients that were obtained by physicians in the Department of Obstetrics & Gynecology, the non-invasive alternative technique to amniocentesis and CVS was further developed. Fingerprinting will help establish whether the isolated cells are free of maternal DNA, which can easily be determined with a blood sample from the mother. With the culmination of this project, there is the hope that the investigated non-invasive method will eventually lead to clinical trials.

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