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Cervical trophoblasts for non-invasive single-cell genotyping and prenatal diagnosis

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ABSTRACT

Objective: We aimed at developing a method to recover trophoblastic cells from the cervix through a completely non-invasive approach and obtaining a genetic proof of their fetal nature implying that they can be used for non-invasive prenatal diagnosis (NIPD).
Methods: We studied obstetrical samples from 21 pregnant women between 8 and 12 weeks of gestation scheduled for chorionic villus sampling or undergoing elective termination of pregnancy. A cytobrush was used to extract cells from the external parts of the cervix and transferred to 10 ml of preservative solution. Cells were layered on filters with 8 microns pores using the ISET system (Isolation by SizE of Tumor/Trophoblastic cells) and stained. Putative fetal cells were collected by single cell laser-assisted microdissection and identified as fetal or maternal cells by Short Tandem Repeat genotyping. NIPD was blindly performed on 6 mothers at risk of having a fetus with Cystic Fibrosis or Spinal Muscular Atrophy.
Results: Trophoblastic cells were recovered from all tested cervical samples with a frequency of 2–12 trophoblasts per 2 ml. NIPD was blindly obtained and verified in 6 mothers at risk of having a fetus with Cystic Fibrosis or Spinal Muscular Atrophy.
Discussion: Although larger confirmation studies are required, this is the first report providing a solid proof of principle that trophoblasts can be consistently and safely recovered from cervical samples. Since they are a source of pure fetal DNA, i.e. fetal DNA not mixed with maternal DNA, they constitute an ideal target to develop NIPD of recessive diseases, which is a technical challenge for methods based on cell free DNA.

1. Introduction

In order to avoid the risk of miscarriage linked to amniocentesis and chorionic villus sampling (CVS) [1], fetal DNA can be, in principle, retrieved non-invasively from three sources: circulating fetal cells in maternal blood [2], transcervical trophoblastic cells [3] and cell-free fetal DNA in maternal blood [4]. The analysis of cell-free fetal DNA has allowed developing reliable non-invasive tests for prenatal detection of aneuploidies [4]. However, the use of cell-free fetal DNA, which is mixed with maternal cell-free DNA in variable proportions, for non-invasive prenatal diagnosis (NIPD) of single-gene disorders and recessive diseases is particularly challenging [5]. In this setting, targeting genetic tests to the pure fetal DNA contained in fetal cells remains an attractive aim.

The presence of fetal cells in the endocervix was first demonstrated by Shettles in 1971 [6]. However, until now the rarity of

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these cells and the difficulty to collect them through a completely non-invasive approach has prevented their implementation for NIPD. Different methods, all collecting fetal cells from the inner part of the cervix and/or the lower pole of the uterine cavity, called transcervical cells (TCC) sampling, were developed, including: intrauterine lavage, endocervical lavage, endocervical mucus aspiration as well as endocervical sampling by a cytobrush [7–18]. Studies have established that uterine and endocervical lavage are the most effective methods to yield fetal cells as early as 5 weeks of gestation [3,7–11]. All these methods however present one major risk: fetal loss [3,19,20]. On the one hand, samples were collected immediately prior to termination of pregnancy in the majority of studies, hence their safety has not been sufficiently examined. On the other hand, establishing the sampling method's safety on a large casistic is difficult for evident reasons.

These observations prompted us to test another approach. We reasoned that the Papanicolaou (PAP) test is currently performed on pregnant women during the first trimester of pregnancy [21], and that its safety has been extensively demonstrated throughout the world. We have thus tested if this sampling approach could consistently detect trophoblastic cells. Interestingly, no study using the PAP-test sampling method to collect cervical trophoblasts had been previously reported.

We also combined this method with our genetic approach to reliably identify trophoblastic cells in blood [22] and avoided the use of antibodies which could lower the sensitivity of trophoblasts detection.

In this study, we aimed at developing a completely non-invasive method to recover trophoblastic cells from the cervix and obtaining a genetic proof of their fetal nature implying that they can be used for non-invasive prenatal diagnosis (NIPD).

2. Materials and methods

We have tested 21 pregnant women (between 8 and 12 weeks of gestation, including 6 (Necker-Enfants Malades Hospital Paris, France), tested immediately before CVS, being at risk for having a baby affected by Cystic Fibrosis or Spinal Muscular Atrophy, and 15 women tested before elective termination of pregnancy (Antoine Béclère, Clamart Hospital, France; Maternity “des Lilas”, Les Lilas, France).

Cells were obtained with the use of a cytobrush, but unlike the reported transcervical cells sampling methods [3], the brush was not inserted into the endocervical canal but rather rotated at the external os, as done during a routine PAP test. Cytobrushes were transferred to 10 ml of a specific preservative solution (Cytofix, Rarecells® Diagnostics, Paris, France). We also obtained 1 ml of blood (collected on EDTA) from each woman and from the father for genomic DNA extraction and testing.

ISET was carried out as previously described [2,22–25] with only minor modifications. In order to layer the cells and eliminate the liquid, 1 ml of each Cytofix sample was diluted 50 fold in bi-distilled, sterile water and subsequently filtered through the Rarecells® Device using a Rarecells® consumable containing an 8 microns pores filter (Rarecells® Diagnostics, Paris, France; www.rarecells.com). The filter was then stained with a 0.1% nuclear fast red stain/5% aluminum sulphate solution (Sigma–Aldrich, St. Louis, MO, USA), incubated for 2 min and then thoroughly rinsed with water. Filters were dried on air.

We became aware of the morphology of cytotrophoblasts by microdissecting putative cytotrophoblasts and analyzing them by short tandem repeat (STR) genotyping. Single cells displaying a cytotrophoblast-like or syncytiotrophoblast-like morphology were retrieved directly from the ISET filters by laser-capture microdissection using the Nikon TE 2000-U (Nikon Paris, France and MMI Zurich, Switzerland) laser-equipped microscope. Each single cell was lifted from the filter and transferred onto the lid of a microfuge tube suited for PCR.

Each microdissected cell was lysed in 15 μL of lysis buffer (100 mmol/L Tris–HCl, pH 8; 400 μg/mL proteinase K) for 2 h at 60 °C, followed by proteinase K inactivation at 94 °C for 15 min. For primer extension preamplification (PEP) [26], to the lysed cell we added 5 μL of a 400 μM solution of random primers (Kit genPEP 75 OD, Genetix, Boston, USA), 6 μL of PCR buffer (25 mM MgCl2/gelatin (1 mg/mL), 100 mM Tris–HCl, pH 8.3, 500 mM KCl), 3 μL of a mixture of four dNTPs (each at 2 mM) and 1 μL (5 U) of Taq polymerase (Applied Biosystem, Foster City, CA, USA) in a final volume of 60 μL. Single-cell genotyping was performed to identify cells having a fetal genome by using STR primers found to be informative through the analysis of paternal and maternal genomic DNA. For genotyping we used 10 different sets of STR genotyping primers from 10 selected STR regions shown in Table 1. Amplification was performed in 60 μL containing 6 μL of the PEP product, 10 mM Tris–HCl, 50 mM KCl, 2.5 mM MgCl2, 200 μM of each deoxynucleotide, 0.5 μM of each STR ‘outer’ primer and 2 U of Taq Gold (Applied Biosystems, Foster City, CA, USA). 2 μl of a 1:10 diluted PCR outer product were re-amplified in a nested PCR in 20 μL final volume using ‘inner’ fluoresceinated STR primers and the same PCR protocol. One μL of the 1:20 diluted inner PCR product (amplicon) was then mixed with 13.5 μL of deionised Hi-Di formamide and 0.5 μL of Genescan 400 HD (ROX) marker (Applied Biosystems) and loaded into an ABI Prism 3100 automated sequencer (Applied Biosystems). Profiles were analyzed using the Genescan and Genotyper software programs (Applied Biosystems).

The NIPD of CF and SMA was performed blindly and carried out as described in other studies [2,24,25].

Invasive diagnoses were carried out at Hôpital Necker-Enfants Malades, Laboratoire de Génétique Médicale, Paris, France.

3. Results

We screened a total of 21 cervical samples from pregnant women between 8 and 12 weeks of gestation, including 6 tested before CVS, at risk for having a baby affected by Cystic Fibrosis or Spinal Muscular Atrophy, and 15 undergoing elective TOP. In all cases cervical samples were obtained by cytobrush and retrieving cells exclusively at the level of the external os, as in the completely safe PAP test. As shown in Fig. 1B, an exocervical squamous epithelial cell (marked with an arrow) is easily morphologically recognized in microscopic images. We were looking for cells displaying a cytotrophoblast-like morphology: round cells with large, irregular hyperchromatic nuclei (Fig. 1B). However, some rare maternal endocervial cells and fetal cytotrophoblasts may have a similar morphology and are therefore much harder to differentiate morphologically (see Fig. 1A and B) without genetic tests. Fetal genotypes were all verified by fluorescent PCR analysis of informative STR markers (Fig. 1C, Table 1). Syncytiotrophoblasts were very rarely found (Table 1). They have dense nuclei and are multinucleated (Fig. 2).

We identified fetal cells (either cytotrophoblasts or cytotrophoblasts and syncytiotrophoblasts) in all 21 samples, with a frequency of 2–12 fetal cells per 2 ml of sample (Table 1). We found approximately 1 cytotrophoblast every two microdissected cells (Table 1).

In order to show that our previously published protocols for NIPD of Cystic Fibrosis and Spinal Muscular Atrophy can be successfully applied to fetal cells isolated from the cervix, NIPD was blindly performed in six cases of pregnant women at risk for having a baby affected by Cystic Fibrosis or Spinal Muscular Atrophy (Table 1). NIPD of Cystic Fibrosis was based on the presence or

Table 1
Isolation of fetal cells from 21 cervical samples obtained non-invasively and exemplary non-invasive prenatal diagnoses (NIPD) of cystic fibrosis (CF) and spinal muscular atrophy (SMA).

Couple	Term of pregnancy (WG)	Informative STR marker	Cytotropho- blasts/Syncytio- trophoblasts* – NIPD**	N° of microdissected cells
1(CF)	12	D7S486/D7S523	4 – carrier	10
2(CF)	12	D7S523	6 – carrier	12
3(CF)	12	D16S539/D7S523	10 – carrier	19
4 (SMA)	12	D5S816/D21S1437	6 – not affected	13
5 (SMA)	12	D21S1435	10 – not affected	21
6 (SMA)	12	D16S539/D7S523	6 – not affected	13
7#	12	D5S816/D21S1437	5	11
8#	12	D16S539	3/2	9
9#	11	D16S539/D5S816	4/2	10
10#	12	D21S1435	10	21
11#	12	D21S1435	6	14
12#	12	D16S3018	7	13
13#	12	D21S1435/D7S523	6	14
14#	12	D16S539/D5S816	2	6
15#	12	D21S11	8/2	16
16#	12	D16S539/D21S1435	12	21
17#	9	D16S3018/D5S615	6	12
18#	9	D5S615/D16S539	4	9
19#	8	D16S539/D5S816	4	10
20#	11	D16S539/D21S11	3	7
21#	8	D5S615/D5S816	3/1	8

*in 2 ml of sample; WG: Week of Gestation; #TOP: Termination of pregnancy; **NIPD was consistent with invasive diagnosis.

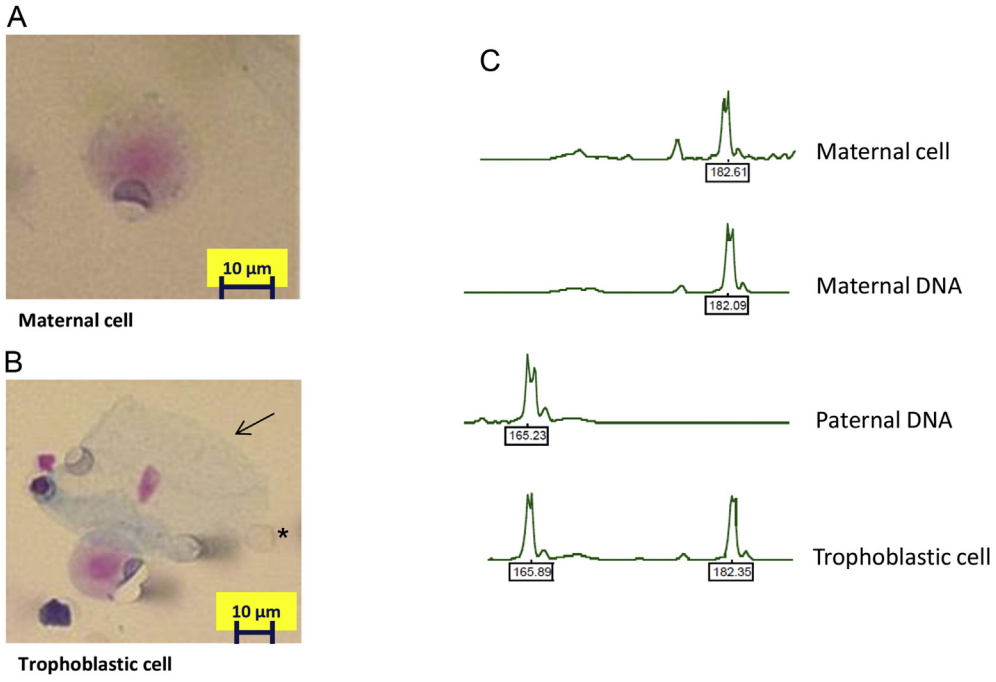

Fig. 1. Detection of a maternal cell (A) and a fetal cell (B) in a sample collected non-invasively from the cervix. Nuclei were colored with nuclear red stain. Marked with an arrow (B) is an exocervical squamous epithelial cell which is easily distinguished from smaller fetal and maternal cells (magnification × 40). An asterisk (B) indicates one of the 8-µm-diameter cylindrical pores on the ISET® filter. Genetic profiles (C), obtained by single-cell genotyping with the informative STR marker D21S1435. Profiles show that the cell in A is maternal, and that the cell in B is fetal (trophoblastic) as compared to the maternal and paternal DNA.

absence of the DelF508 mutated allele(s) as both parents in the 3 couples (Couples 1, 2 and 3, Table 1) were carrier of the DelF508 mutated allele [2,25]. We determined that all 3 fetuses were carriers of the DelF508 mutation. NIPD of Spinal Muscular Atrophy was carried out in fetal cells from couples 4, 5, 6 (Table 1) using our previously published method [2,24]. We determined that none of the 3 fetuses was affected by Spinal Muscular Atrophy. Our analyses were performed blindly and results were consistent with those obtained by invasive prenatal diagnosis performed after CVS.

4. Discussion

Our results show that trophoblasts, (also called trophectodermal cells) were recovered non-invasively from all tested 21 pregnant women and allowed blind NIPD in 6 mothers at risk of having a fetus with Cystic Fibrosis or Spinal Muscular Atrophy. The goal of our study was to determine if trophoblastic cells could be consistently isolated from the cervix using a completely safe sampling method. This goal implied using the PAP-test sampling approach,

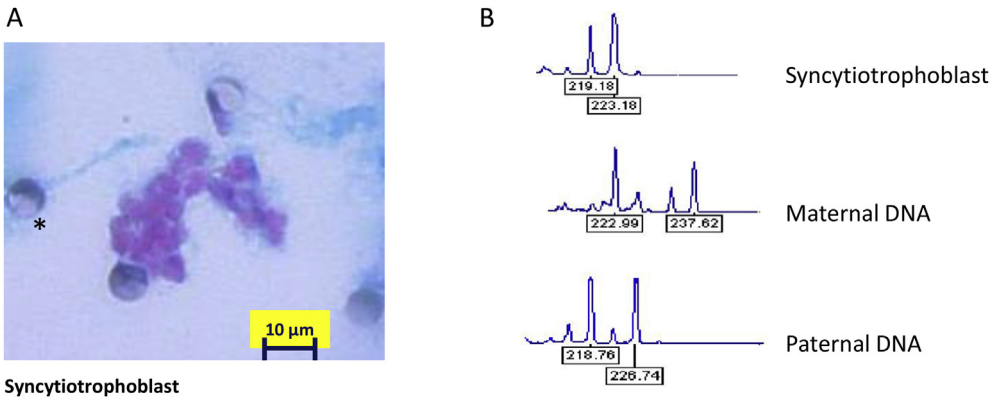

Fig. 2. Detection of a syncytiotrophoblast (A) in a sample collected non-invasively from the cervix. Nuclei were colored with nuclear red stain (magnification × 40). Its genetic profile obtained through STR-genotyping with the D21S11 STR marker is fetal (trophoblastic) as compared to the maternal and the paternal DNA (B).

layering the collected cells on a filter by ISET, using cell morphology to target cells eligible for individual genotyping and identifying every trophoblastic cell by STR genotyping. No study had been performed in the past using a completely non-invasive collection method [3], proven to be safe by previous extensive and routine use. This is the case for the PAP-test sampling method collecting cells from the exocervix, which has been routinely performed on pregnant women for several decades [21].

Trophoblastic cells are thought to be shed from regressing chorionic villi in the uterine cavity, which disappears between 11 and 12 weeks of gestation following the fusion of the *decidua basalis* and *parietalis*, and from it towards the cervix [6,27]. Hence the possibility of collecting trophoblastic cells from cervix is expected to be transient and restricted to early terms of pregnancy.

We treated the samples with ISET, a technology developed in our laboratory, which layers the cells onto a membrane and poises them for single cell microdissection followed by STR genotyping. Using this approach, we were able to demonstrate the collection of trophoblastic cells in all tested cases through the analysis of only 2 ml of each 10 ml sample. We believe that the success of our approach is linked to several factors: we decided arbitrarily 1) to collect the cells using the PAP-test sampling approach, to ensure safety; 2) to filtrate the cells, to avoid their overlapping (and loss of detection of rare cells); 3) not to use antibodies to identify trophoblastic cells, to avoid loss of sensitivity; 4) to verify the fetal nature of putative trophoblasts through single cell STR genotyping.

We did not use antibodies to identify trophoblastic cells as we were concerned by the fact that these cells are very rare and that the use of antibodies would probably lead to the loss of some of them. In fact, it is thought that identification of fetal cells through immunological labeling is exposed to the risk of false positive and false negative results [28,29]. In the past several studies have used antibody staining and/or cell morphology without validation by molecular testing [11,30–32]. Two groups used immunolabelling with NDOG-1 antibody in combination with genetic analyses [18,33]. Whereas one of them [33] reported that only half of their samples contained fetal cells, the other showed detection of fetal cells in all 22 screened samples with a frequency of 5–10 fetal cells per sample [18] and found syncytiotrophoblasts in 116 of 207 (56%) transcervical cells (TCC) samples [18]. However, samples were obtained through endocervical mucus aspiration, a semi-invasive procedure carried out under general anesthesia immediately prior to termination of pregnancy. In other studies, employing antibodies anti-HLA-G [32,34], samples were obtained with the least, but still invasive transcervical cells sampling method, using a cytobrush inserted about 2 cm into the endocervical canal to retrieve cervical mucus. Although no direct adverse effects were observed, it can be imagined that pregnant women would be reluctant towards such transcervical cells sampling. Imundia et al. [32] identified putative cytotrophoblasts in 35 out of 37 transcervical cells samples but did not confirm their fetal nature by genetic analysis. Bolnick et al. [34] used Y chromosome Fluorescence *In Situ* Hybridization (FISH) to validate the fetal origin of 99% of the cells they had retrieved. But since their FISH approach could only confirm fetal origin of trophoblastic cells retrieved from male fetuses, the author did not extend the study to the 9 female specimens identified amongst the 20 pregnancies tested.

Choosing a different and entirely non-invasive approach, we used cell morphology to select cells for further genotyping analyses. In fact, we learnt about the morphology of cytotrophoblasts by microdissecting putative cytotrophoblasts and analyzing them by STR genotyping. We observed undeniably that fetal cells are generally smaller and have a smaller cytoplasm when compared to the majority of cervical maternal cells. By using this approach we found 2 to 12 fetal cells per 2 ml of a 10 ml sample, potentially 10 to 60 trophoblastic cells per cervical sample.

Our pilot study shows that trophoblastic cells are located at the external zone of the cervix and can be consistently recovered by the PAP test sampling approach. This is a very encouraging news for collecting these precious rare cells without any risk for the fetus.

In summary, we have performed an original study which provides a proof of principle for consistent and safe collection of trophoblastic cells from cervical samples at an early term of pregnancy and for their use in NIPD of recessive diseases. Although these results require a larger scale study to obtain further confirmation, this is the first report on a completely safe and successful approach to isolate trophoblastic cells from cervix aiming at non-invasive prenatal diagnosis testing. In particular, antibodies reported to be specific to trophoblastic cells should be tested in parallel with our approach not relying on antibodies to assess their efficiency and sensitivity for trophoblastic cells identification. This is clearly a key issue related to the cost of a potential future test and its possible “routinisation” [5]. However, exploring the diagnostic potential of fetal cells collected non-invasively seems a clinically interesting path, which could benefit from recent advances showing the use of Next Generation Sequencing for single blastomers' molecular analyses [35].

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