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Trophoblast retrieval and isolation from the cervix: origins of cervical trophoblasts and their potential value for risk assessment of ongoing pregnancies

Gerit Moser[1,†], Sascha Drewlo[2,†], Berthold Huppertz[1], and D. Randall Armant[3,*]

[1]Department of Cell Biology, Histology and Embryology, Gottfried Schatz Research Center, Medical University of Graz, Neue Stiftingtalstrasse 6/II, 8010 Graz, Austria [2]Department of Obstetrics, Gynecology and Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, MI 49503, USA [3]Department of Obstetrics and Gynecology, Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI 48201, USA

*Correspondence address. C.S. Mott Center for Human Growth and Development, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, 275 East Hancock Street, Detroit, MI 48201-1405, USA. Tel: +1-313-577-1748; Fax: +1-313-577-8554; E-mail: d.armant@wayne.edu orcid.org/0000-0001-5904-9325

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[†]These authors are contributed equally to this work.

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BACKGROUND: Early during human development, the trophoblast lineage differentiates to commence placentation. Where the placenta contacts the uterine decidua, extravillous trophoblast (EVT) cells differentiate and invade maternal tissues. EVT cells, identified by expression of HLA-G, invade into uterine blood vessels (endovascular EVT), as well as glands (endoglandular EVT), and open such luminal structures towards the intervillous space of the placenta. Endoglandular invasion diverts the contents of uterine glands to the intervillous space, while glands near the margin of the placenta that also contain endoglandular EVT cells open into the reproductive tract. Cells of the trophoblast lineage have thus been recovered from the uterine cavity and endocervical canal. An emerging non-invasive technology [trophoblast retrieval and isolation from the cervix (*TRIC*)] isolates and examines EVT cells residing in the cervix to explore their origin, biology and relationship to pregnancy and fetal status.

OBJECTIVE AND RATIONALE: This review explores the origins and possible uses of trophoblast cells obtained during ongoing pregnancies (weeks 5–20) by *TRIC*. We hypothesize that endoglandular EVT cells at the margins of the expanding placenta enter the uterine cavity and are carried together with uterine secretion products to the cervix where they can be retrieved from a Papanicolaou (Pap) smear. The advantages of *TRIC* for investigation of human placentation and prenatal testing will be considered. Evidence from the literature, and from archived *in utero* placental histological sections, is presented to support these hypotheses.

SEARCH METHODS: We used 52 out of 80 publications that appeared between 1966 and 2017 and were found by searching the PubMed and Google Scholar databases. The studies described trophoblast invasion of uterine vessels and glands, as well as trophoblast cells residing in the reproductive tract. This was supplemented with literature on human placental health and disease.

OUTCOMES: The literature describes a variety of invasive routes taken by EVT cells at the fetal–maternal interface that could displace them into the reproductive tract. Since the 1970s, investigators have attempted to recover trophoblast cells from the uterus or cervix for prenatal diagnostics. Trophoblast cells from Pap smears obtained at 5–20 weeks of gestation have been purified (>95% β-hCG positive) by immunomagnetic isolation with nanoparticles linked to anti-HLA-G (*TRIC*). The isolated cells contain the fetal genome, and have an EVT-like expression profile. Similar EVT-like cells appear in the lumen of uterine glands and can be observed entering the uterine cavity along the margins of the placenta, suggesting that they are the primary source of cervical trophoblast cells. Cells isolated by TRIC can be used to accurately genotype the embryo/fetus by targeted next-generation sequencing. Biomarker protein expression quantified in cervical trophoblast cells after *TRIC* correlates with subsequent pregnancy loss, pre-eclampsia and fetal growth restriction. A key remaining question is the degree to which EVT cells in the cervix might differ from those in the basal plate and placental bed.

WIDER IMPLICATIONS: *TRIC* could one day provide a method of risk assessment for maternal and fetal disease, and reveal molecular pathways disrupted during the first trimester in EVT cells associated with placental maldevelopment. As perinatal interventions emerge for pregnancy disorders and inherited congenital disorders, *TRIC* could provide a key diagnostic tool for personalized precision medicine in obstetrics.

Key words: trophoblast / pregnancy / prenatal diagnosis / female reproductive tract / genetic diagnosis / endometrium / immunohistochemistry / placenta / obstetric disorders

Introduction

Pregnancy in humans, while most often producing a favorable outcome, is subject to significant morbidity and mortality to the mother, fetus and resulting child characterized by multiple congenital anomalies and clinical syndromes (Di Renzo, 2009; Romero, 2009). Because the fetus and placenta are nearly inaccessible during pregnancy, predicting adverse outcomes is challenging without the use of invasive approaches such as amniocentesis or chorionic villous sampling (CVS). Biomarkers in the maternal circulation have been explored for screening in the first trimester, but so far are only adequately reliable to be considered diagnostic for a small proportion of pregnancy pathologies. Identification of at-risk pregnancies early in gestation offers the best opportunities to discover etiologies and develop corrective interventions. A position paper by senior staff of the National Institute of Child Health and Human Development in the USA points out that the knowledge barrier to understanding the mechanisms associated with feto-placental disorders 'is primarily due to limitations in current non-invasive "interrogation" of the placenta' (Guttmacher *et al.*, 2014).

For years, circulating protein biomarkers combined with ultrasonic monitoring have been used to detect neural tube defects and chromosomal abnormalities, as well as for determining risk for pregnancy disorders such as early-onset pre-eclampsia and other placental maldevelopment syndromes (SMFM, 2016). Various combinations of molecules accumulate in maternal blood that are indicative of placental maldevelopment-associated pathologies, including pregnancy-associated plasma protein A (PAPP-A), placental protein 13 (LGALS13 or PP13), inhibin, hCG, placental growth factor (PlGF or PGF) and soluble fms-like tyrosine kinase-1 (FLT1) (Wright *et al.*, 2017). While these biomarkers have been used in combination with sonographic and Doppler velocimetry methods in the first and second trimesters, there are significant limitations in terms of their clinical utility based on their low sensitivity and positive predictive values for detection of small for gestational age newborns (ACOG, 2015; McCowan *et al.*, 2017; Parry *et al.*, 2017).

A promising approach for interrogating the placenta that is the subject of this review utilizes trophoblast cells that migrate from the placenta into the reproductive tract (Imudia *et al.*, 2010). It is possible to recover hundreds of placental cells safely and non-invasively from the endocervical canal using a Papanicolaou (Pap) procedure (Canestrini and Testa, 1978; Fejgin *et al.*, 2001; Cioni *et al.*, 2005; Imudia *et al.*, 2009), and then separate them from maternal cells for downstream analysis of fetal DNA, RNA, proteins and other informative molecules (Drewlo and Armant, 2017).

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Methods

The PubMed and Google Scholar databases were comprehensively searched for relevant publications describing the migration of trophoblast cells into endometrial glands and the uterine lumen, as well as investigations of trophoblast cells residing in the reproductive tract and cervix. The publications identified between 1966 and 2017 included those published on cervical trophoblast from the time fetal cells were first found in Pap smears, as well as those exploring subsets of trophoblast in endometrial arterioles, venules and glands. Publications were identified by searching with combinations of the keywords, human, cervix, endocervical canal, cervical mucus, trophoblast, fetal cells, pregnancy, transcervical sampling, retrieval, isolation, DNA, genome, extravillous, invasion, glands, arteries, veins. Eighty publications were reviewed and 52 were selected that reported significant findings on the topic of trophoblast cells residing in the uterine cavity and cervix. An additional 69 references with keywords related to human placental health and disease were integrated into the review to expand on the topic and provide pertinent background information.

Trophoblast cells in maternal blood and the reproductive tract

The prospect of prenatal diagnosis with less risk than amniocentesis or CVS has been sought since it came to light that fetal cells are present in accessible locations, including maternal blood and the reproductive tract. The presence of Y chromosome-containing fetal cells deported into the maternal circulation has been recognized since the early 1980s (Iverson *et al.*, 1981), and in the reproductive tract since the 1970s (Shettles, 1971).

Non-invasive prenatal testing using maternal blood samples

While successes were reported with isolation of fetal trophoblast and hematopoietic cells from the maternal circulation, their numbers were challenging (on the order of 1 per million maternal cells), and the field shifted toward the exploitation of cell-free fetal DNA (Norwitz and Levy, 2013; Wou *et al.*, 2015). Non-invasive prenatal testing (NIPT), using cell-free fetal DNA in maternal blood, can screen for the most common chromosome number disorders (13, 18, 21, X, Y) beginning around 10 weeks of pregnancy (Bianchi *et al.*, 2014). Cell-free fetal DNA is a slight misnomer, as it is derived from turnover of tissue in the placenta, rather than the fetus. The fetal, or placental, fraction must exceed 4% of the total cell-free DNA in maternal plasma to be reliably sequenced and interpreted (Wong and Lo, 2016). Although detection of trisomies 13, 18 and 21 has been impressive (98–99%) with false positive rates of ~0.13% (Gil *et al.*, 2017), NIPT has not been accepted for detection of single gene mutations. Moreover, trisomies observed through NIPT screening should be confirmed by invasive diagnostic testing (Benn *et al.*, 2015; Gregg *et al.*, 2016). It has been reported that NIPT has high sensitivity and specificity for detection of paternally contributed genetic traits, such as sex and Rhesus D antigen in D-negative women (Devaney *et al.*, 2011; Johnson *et al.*, 2017), and the possibility exists for its eventual use as a diagnostic tool (Drury *et al.*, 2016).

Trophoblast cells in the female reproductive tract and cervical canal

Subsequent to the initial observation of Shettles (1971), a number of investigators attempted to replicate and extend the transcervical approach for obtaining intact placental cells, not only in cervical smears or aspirated mucus, but also through more invasive endocervical and intrauterine lavage (Adinolfi and Sherlock, 1997, 2001; Bischoff and Simpson, 2006; Evans and Kilpatrick, 2010; Imudia *et al.*, 2010; Wou *et al.*, 2015). Early attempts during the 1970s to repeat Shettles' findings were met with both success (Rhine *et al.*, 1975) and failure (Bobrow and Lewis, 1971; Manuel *et al.*, 1974; Amankwah and Bond, 1978; Goldberg *et al.*, 1980). The reliability of Y-body fluorescence produced with quinacrine mustard to detect male cells, as used by Shettles, was the principal impediment to acceptance of the approach and further advancement at that time.

Attention turned away from exploitation of trophoblast cells in the reproductive tract for a decade, but then resumed to produce some important advances that are outlined in Table I. With the development of fluorescence *in situ* hybridization (FISH) and PCR, and the realization that adequate numbers of fetal cells for prenatal testing were unlikely to be available in maternal blood, interest returned during the 1990s to trophoblast cells residing in the uterus and endocervical canal (Griffith-Jones *et al.*, 1992; Pertl *et al.*, 1994; Ville *et al.*, 1994; Briggs *et al.*, 1995; Adinolfi *et al.*, 1995c; Fung *et al.*, 1995; Ishai *et al.*, 1995; Kingdom *et al.*, 1995; Rodeck *et al.*, 1995). Using FISH and PCR, success in determining fetal sex from transcervical specimens was variable, but encouraging. An approach using X22 heterozygosity to distinguish the maternal genome from a female fetal genome was reported (Adinolfi and Cirigliano, 2000). Trophoblast protein markers and morphological assessment also identified trophoblast cell populations within specimens collected transcervically (Chaouat *et al.*, 1994; Bahado-Singh *et al.*, 1995; Bulmer *et al.*, 1995). Early successes demonstrated the potential of transcervical sampling for clinical diagnosis including the detection of fetal trisomy 18 by FISH (Adinolfi *et al.*, 1993), fetal rhesus blood group D antigen gene in rhesus negative patients (Adinolfi et al., 1995a), fetal chromosome 21 using both FISH and chromosome-specific short tandem repeat (STR) amplification (Adinolfi *et al.*, 1995b), fetal chromosome number variants by FISH and PCR (Massari *et al.*, 1996; Sherlock *et al.*, 1997; Xi Zhao *et al.*, 2003; Bussani *et al.*, 2007), fetal hemoglobin genotypes responsible for sickle cell disease and thalassemia (Adinolfi *et al.*, 1997; Cirigliano *et al.*, 1999), and fetal triple X (Xi Zhao *et al.*, 2003).

Genetic interrogation of trophoblast cells obtained transcervically relied principally upon morphological identification and micromanipulation or the use of trophoblast-specific markers (Adinolfi and Sherlock, 2001), limiting its usefulness in a clinical setting. Efforts to develop antibodies or antibody panels to distinguish trophoblast cells in transcervical specimens were undertaken during this period (Chaouat *et al.*, 1994; Bulmer *et al.*, 1995). Laser capture microdissection after identification of trophoblast cells by immunostaining with antibodies to selective lineage markers successfully provided fetal DNA for PCR genotyping (Bulmer *et al.*, 2003; Mantzaris and Cram, 2015). Some success in DNA allelic profiling by single-cell approaches has been reported (Katz-Jaffe *et al.*, 2005; Pfeifer *et al.*, 2016), but these advances have not led

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Table I Key findings, from 1971 to 2016, using transcervical retrieval of trophoblast cells.

Advancements (chronological)	Retrieval method	Trophoblast detection method	References
Trophoblast cells found in cervix	Cervical swab smeared on slide	Y-body stain with quinacrine mustard	Shettles (1971)
Confirmation of cervical trophoblast cells	Cervical swabs smeared on slide	PCR for Y-derived sequences	Griffith-Jones *et al.* (1992)
Identification of syncytial trophoblast fragments and protein markers	Uterine lavage	Histology and immunocytochemistry	Griffith-Jones *et al.* (1992); Chaouat *et al.* (1994)
Detection of fetal trisomy	Uterine lavage	FISH for chromosomes 18 and Y	Adinolfi *et al.* (1993)
Fetal RHD detected in Rh negative women	Cervical mucus aspiration and uterine lavage	PCR	Adinolfi *et al.* (1995a)
Paternal STR allele detection	Endocervical lavage and mucus aspiration	PCR-seq to detect STRs	Adinolfi *et al.* (1995b); Kingdom *et al.* (1995)
Trisomy 21 detected by STR analysis	Uterine lavage, mucus aspiration and cytobrush	PCR-seq for STRs	Adinolfi *et al.* (1995c)
Detection of fetal SMA and myotonic dystrophy	Endocervical lavage	PCR-seq for STRs	Massari *et al.* (1996)
Trophoblast enrichment by morphology	Endocervical collection and micromanipulation based on morphology	FISH and PCR for Y chromosome	Sherlock *et al.* (1997)
Detection of fetal hemoglobinopathies	Cervical mucus aspiration and micromanipulation based on morphology	PCR-seq for STRs and hemoglobin mutations	Adinolfi *et al.* (1997); Cirigliano *et al.* (1999)
Identification of female trophoblast cells	Cervical mucus aspiration	PCR-seq for X22	Adinolfi and Cirigliano (2000)
Reliable (97%) cervical trophoblasts recovery	Endocervical collection by cytobrush	FISH	Fejgin *et al.* (2001)
LCM isolation of trophoblasts	Uterine lavage, labeled with anti-HLA-G for LCM	PCR-seq for X, Y and paternal STRs	Bulmer *et al.* (2003)
Trophoblast enrichment with marker antigens	Immunofluorescence labeling and isolation by micromanipulation	PCR-seq for STRs	Katz-Jaffe *et al.* (2005); Mantzaris *et al.* (2005)
Enrichment of trophoblast based on morphology (152/181)	Uterine lavage and micromanipulation	PCR-seq for STRs	Bussani *et al.* (2007)
Reduced numbers of cervical trophoblast in pathological pregnancies	Endocervical collection by cytobrush	Immunocytochemistry for HLA-G	Imudia *et al.* (2009)
Isolation of >900 EVT cells to >95% purity from Pap specimens by TRIC	Endocervical collection by cytobrush	Immunomagnetic isolation of trophoblast (EVT) with anti-HLA-G	Bolnick *et al.* (2014)
EVT isolation by TRIC is unaltered by gestational age at 5–20 weeks or BMI	Endocervical collection by cytobrush and *TRIC*	Immunocytochemistry for HLA-G	Fritz *et al.* (2015b)
Trophoblast isolation by size and LCM	External cervical collection by cytobrush and ISET/LCM	Single cell STR genotyping	Pfeifer *et al.* (2016)
EVT protein profiles reflect pregnancy outcomes	Endocervical collection by cytobrush and *TRIC*	Immunocytochemistry for biomarkers of placental insufficiency	Fritz *et al.* (2015a); Bolnick *et al.* (2016b)
DNA profiling of EVT by NGS	Endocervical collection by cytobrush and *TRIC*	Fetal DNA isolation and targeted NGS for SNPs and STRs	Jain *et al.* (2016)

STR, short tandem repeats; PCR-seq, Sanger sequencing of fluorescence-labeled PCR products; SMA, spinal muscular atrophy; LCM, laser capture microdissection; EVT, extravillous trophoblast; *TRIC*, trophoblast retrieval and isolation from the cervix; ISET, isolation by size of epithelial tumor; NGS, next-generation sequencing; SNP, single nucleotide polymorphism; RHD, rhesus blood group D antigen.

to a consistent and efficient procedure that could be translated into clinical practice. Success rates for obtaining trophoblast cells remained variable and largely unacceptable for prenatal testing regardless of the invasiveness of the method used to obtain specimens (Ergln *et al.*, 2001; Fejgin *et al.*, 2001; Cioni *et al.*, 2003; Mantzaris *et al.*, 2005; Bussani *et al.*, 2007; Mantzaris and Cram, 2015). However, it was also clear from these investigations that during the first trimester trophoblast cells indeed reside freely in the maternal reproductive tract despite its structural separation from the chorionic villi within the chorion laeve and decidua capsularis.

Routes for trophoblast migration into the reproductive tract

If it is hypothesized that trophoblast cells can be retrieved from the reproductive tract during early pregnancy, a rationale is needed for their displacement from the placenta. At the tips of anchoring villi, cytotrophoblast cell columns attach to the maternal decidua and differentiating EVT cells invade the maternal tissues. Thereafter, they follow the interstitial route of invasion. Further routes of EVT invasion

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based on interstitial trophoblast cells include the uterine blood vessels (endovascular EVT subdivided into endoarterial EVT in uterine arteries and endovenous EVT in uterine veins) and uterine glands (endoglandular EVT) (Moser and Huppertz, 2017). The endovascular and endoglandular EVT activities are responsible for the tissue remodeling that establishes the hemotrophic and histiotrophic nutrition of the fetus and embryo, respectively (Benirschke et al., 2012a; Moser et al., 2015). Within the trophoblast cell column, cells express HLA-G as they differentiate from villous to extravillous, which parallels the physiological change from proliferative (HLA-G negative) to invasive (HLA-G positive) properties, and correlates with a switch in the expression of integrin subunits that mobilizes the cells (Merviel et al., 2001; Benirschke et al., 2012b). HLA-G expressing EVT cells can be visualized in the decidua basalis within the stroma, within vessels and within uterine glands, and occasionally in the uterine cavity (Fig. 1). HLA-G is a reliable phenotypic marker of differentiated EVT (McMaster et al., 1995; Apps et al., 2008; Moser et al., 2011), and is also expressed by trophoblast cells residing in the endocervical canal (Imudia et al., 2009).

During placental development, there are two possible routes that EVT cells could take to reach the uterine cavity and, subsequently, the cervix. First, interstitial EVT cells expressing HLA-G penetrate the uterine epithelium near the margin of the placenta, replace the uterine epithelium from the basal side, and enter the uterine cavity (Fig. 2). Second, at the edge of the developing placenta, in the transitional zone of decidua basalis and decidua parietalis, endoglandular EVT cells invade uterine glands from the basal side, replace the glandular epithelium, and occupy the glandular lumen (Moser et al., 2010, 2015). Eroded uterine glands can be visualized (Fig. 3a and b) especially at the margin of the developing placenta (Moser and Huppertz, 2017). The developing placenta rapidly expands laterally (Craven et al., 2000; Nanaev et al., 2000), continuously exposing new glands to EVT invasion at the margin of the placenta. These invaded glands can secrete further laterally into the uterine cavity (Fig. 2), and thus could expel EVT cells together with the glandular secretion products into the uterine cavity. After they reach the uterine cavity, EVT cells could be carried together with the secretion products toward the cervix, as illustrated in Fig. 4.

Interstitial, as well as endovascular and endoglandular, EVT cells react with antibodies against the pro-invasive proteins matrix metalloproteinase 1 (MMP1) (Weiss et al., 2016) and integrin β1 (ITGB1) (Kemp et al., 2002), which is shown in Fig. 3c–f. The expression of these molecules points to the invasive capacity, and thus to a common (extravillous) origin of the cells. The migratory potential of the EVT was established decades ago (Billington, 1966), supporting the hypothesis that EVT cells migrate from the uterine cavity toward the cervix. The developing placenta expands rapidly, hence, there could be a continuous supply of EVT cells entering the endocervical canal.

As the placenta expands to fill the uterine cavity, it would be expected that migration of EVT cells into the cervix would cease. Accordingly, it is observed that endocervical EVT cells are recovered in Pap specimens only until 20 weeks of gestational age (GA), after which cell recoveries rapidly plummet in the authors' (D.R.A. and S. D.) experience (Fritz et al., 2015b). Whether trophoblast cells not expressing HLA-G remain longer in the cervix is unclear.

As indicated by the literature cited in Table I, there are numerous reports of syncytial trophoblast, and even villous fragments, recovered through transcervical collections. It has been suggested that ulceration of the decidua capsularis might permit release of these elements into the uterine cavity (Adinolfi and Sherlock, 2001). Direct evidence for this route is limited, and the inconsistencies reported for retrieval of villous trophoblast elements after uterine or endocervical lavage (Bahado-Singh et al., 1995; Miller et al., 1999; Fang et al., 2005; Bussani et al., 2007) suggest that it might not be a natural route. Further investigation is needed to resolve this issue.

Trophoblast Retrieval and Isolation From the Cervix

Technical issues of *TRIC*

Although endocervical specimens from pregnant women could provide useful information to clinicians, the approach initially did not prove robust, due fully or in large part to the excess of maternal cells from which trophoblast cells must be distinguished for efficient and accurate analysis (Imudia et al., 2010). Trophoblast retrieval and isolation from the cervix (*TRIC*) was developed as a solution to the technical challenge of analyzing placental cells in real time, leveraging the unique expression of HLA-G on the surface of EVT cells, exclusive of adult tissues in the reproductive tract (Loke et al., 1997; McMaster et al., 1998), to obtain a homogeneous population of fetal cells with an EVT-like phenotype (Bolnick et al., 2014). The *TRIC* procedure separates trophoblast cells from maternal cells using immunomagnetic nanoparticles, to provide placental cells for downstream molecular analyses. The information that *TRIC* provides could complement data obtained using current NIPT procedures that are based on biophysical and biochemical measurements.

Retrieval of trophoblast cells from the endometrial canal using a cytobrush can be considered minimally invasive. The office procedure is essentially a Pap smear, and can be performed successfully between 5 and 20 weeks GA (Fritz et al., 2015b). Pap smears are recommended during pregnancy, and several studies that surveyed approximately 1900 pregnant women found the cytobrush procedure to be safe and associated with no serious adverse outcomes (Orr et al., 1992; Rivlin et al., 1993; Paraiso et al., 1994; Holt et al., 2005). Our (S.D. and D.R.A.) experience with over 1000 endocervical samples collected from ongoing pregnancies has revealed no increase above baseline frequencies in pregnancy loss, excessive bleeding or infection (manuscript in preparation).

Analytical issues of *TRIC*

The isolation of EVT cells by *TRIC* is enhanced with inclusion of adequate quality controls. Ideally, endocervical specimens will contain 1 000 000 or more cells, determined in a simple cell count, although specimens with as few as 50 000 cells can provide highly purified trophoblast cells. However, yield will be affected by the initial cell number, since there are approximately 2000 maternal cells for every trophoblast cell (Imudia et al., 2009). An aliquot containing 5–10% of the endocervical specimen should be examined by immunocytochemical labeling with anti-HLA-G to provide an estimate of the number of EVT cells that can be expected in the final isolate (Bolnick et al., 2014). Trophoblast cells are also distinct from the maternal cells in their expression of β-hCG, which can be used to determine the

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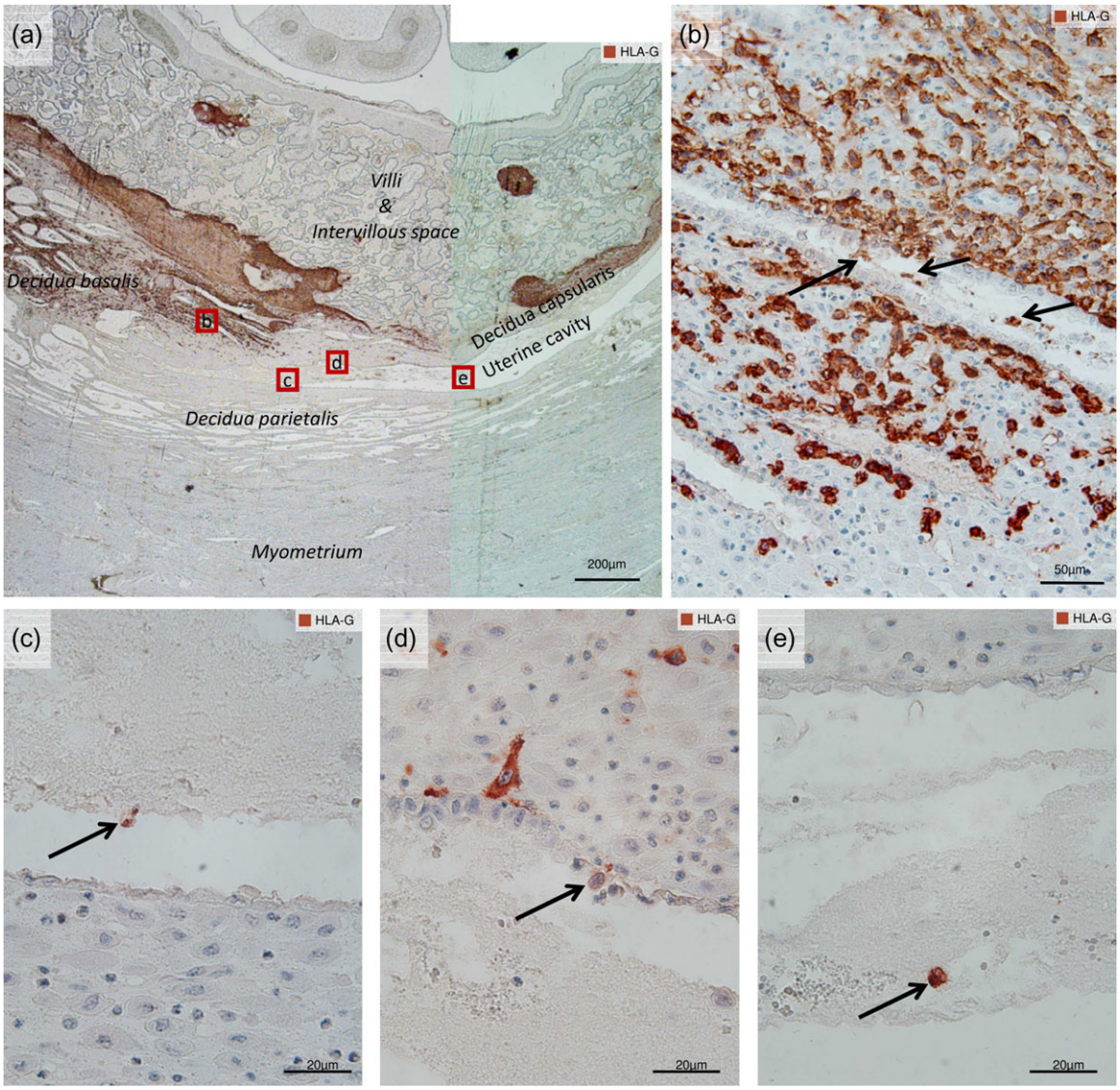

Figure 1 Extravillous trophoblast cells in the human uterine cavity. Immunocytochemical staining *in utero* with an antibody against HLA-G (and Hemalaun nuclear counterstain) in paraffin sections of an archived placenta (most likely early first trimester). The dark brown labeling of HLA-G serves as a marker for extravillous trophoblast (EVT) cells in the invasive zone between fetal and maternal regions. (**a**) An overview at the margin of the placenta showing villi and intervillous space, decidua basalis, decidua parietalis, decidua capsularis and the uterine cavity, as labeled. Details of the red insets in (**a**) follow: (**b**) demonstrates endoglandular EVTs (arrows) in the lumen of a gland near the edge of the placenta. (**c**) Shows an HLA-G positive EVT cell (arrow) located in the uterine cavity. (**d**) Shows an EVT cell (arrow) that has replaced the uterine epithelium, while others nearby approach the epithelium. (**e**) Shows another EVT cell located in the uterine cavity, possibly surrounded by glandular secretions.

purity of the final isolate. Typically, 95–99% of the cells isolated by *TRIC* are β-hCG positive, but some samples range lower, down to 75–85% (Bolnick *et al.*, 2014; Fritz *et al.*, 2015b). Trophoblast cells obtained by *TRIC*, when examined by FISH or single-cell PCR for the X and Y chromosomes, consistently demonstrate either XY or XX signals, in agreement with the sex of the babies delivered. Moreover, cells isolated from male specimens are seldom XX, verifying the fetal origin and purity of cells obtained by *TRIC* (Bolnick *et al.*, 2014). Conversely, the cells excluded during immunomagnetic isolation are uniformly female and β-hCG negative, as expected of the maternal cells in the cervix.

Specificity of *TRIC*

Cells obtained by *TRIC* have been profiled by immunocytochemistry to characterize their lineage and phenotype. The trophoblast marker proteins cytokeratin 7 and placental lactogen (CSH1) are uniformly expressed in the isolated cell fraction, in addition to β-hCG (Bolnick *et al.*, 2014). Because *TRIC* employs affinity to HLA-G, a protein associated with EVT differentiation, selective isolation of that trophoblast population would be expected. As expected, the isolated cells express several EVT markers (integrin subunit α1 [ITGA1], cadherin 5, platelet and endothelial cell adhesion molecule 1, MMP9, HLA-G),

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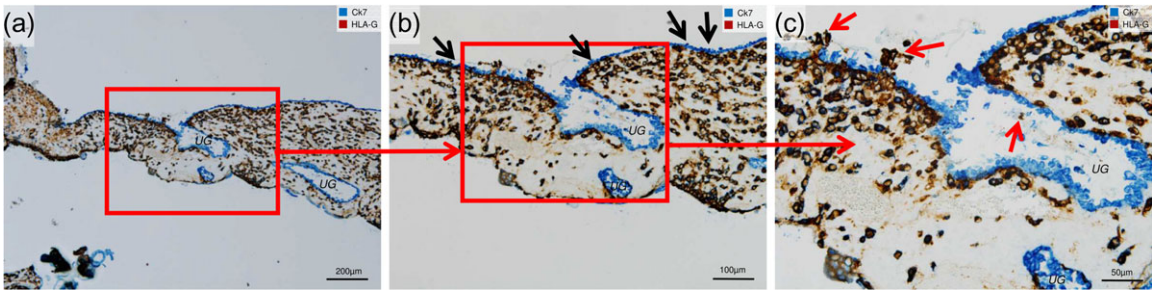

Figure 2 EVT cells replace the uterine epithelium. Immunocytochemical double staining of invaded decidua (7 weeks gestational age) for cytokeratin 7 (Ck7, blue, serves as marker for glandular and uterine epithelium) and HLA-G (dark brown, serves as marker for EVT). No nuclear counterstain. (**a**) Overview shows the transitional zone between decidua capsularis (to the left) and decidua basalis (to the right) with uterine cavity above and intervillous space below. The decidua basalis includes prominent uterine glands (UG) with blue-labeled epithelia. (**b**) Inset shown in (**a**). Black arrows indicate the uterine epithelium. (**c**) Inset shown in (**b**). Higher magnification shows EVT cells (red arrows) breaking through the uterine epithelium at the opening of the UG, and an endoglandular EVT cell in the lumen of the gland.

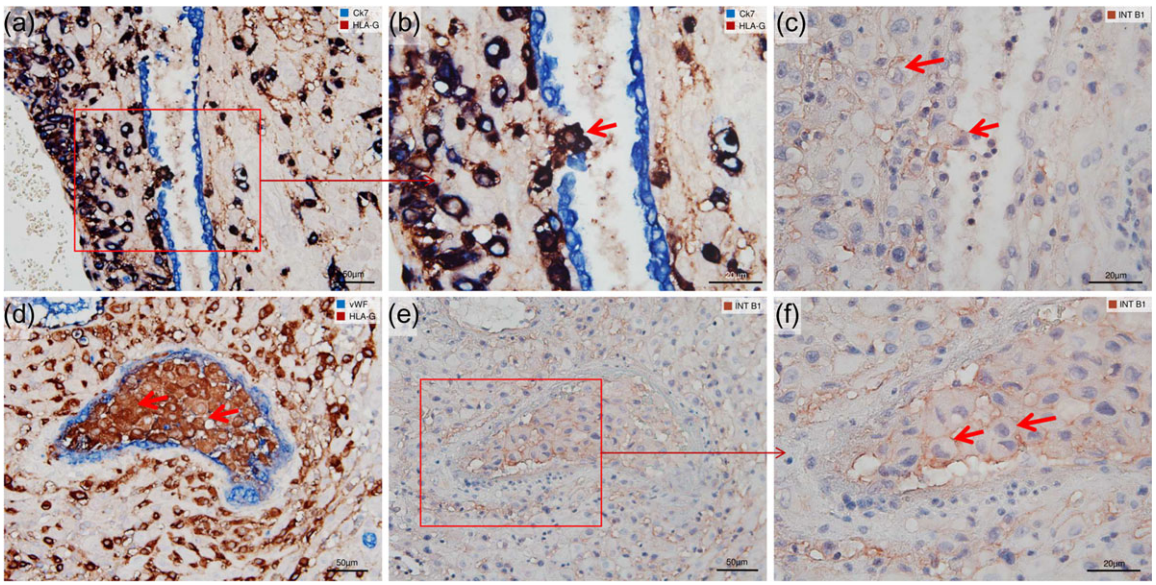

Figure 3 Endoglandular and endovascular trophoblast cells express integrin ITGB1. Immunocytochemical single and double staining of serial sections of invaded decidua (11 weeks gestational age) for integrin subunit ITGB1 (INT B1, brown), CK7 (blue, serves as marker for glandular and uterine epithelium), HLA-G (dark brown, serves as marker for EVT), and von Willebrand Factor (vWF, blue, serves as marker for vascular endothelium). Colors of the labels are indicated in each panel. No nuclear counterstain in (**a**, **b**, **d**). Nuclei were counterstained with Hemalaun in (**c**, **e**, **f**). (**a**) Overview showing a UG partly surrounded by EVT, while in (**b**) a higher magnification of inset shown in (a) clearly demonstrates endoglandular EVT cells (red arrow) breaking through the glandular epithelium towards the glandular lumen. (**c**) Adjacent section to (**b**) labeled for ITGB1. Like the interstitial EVT, the endoglandular EVT (red arrows) express ITGB1 on their cell surface. (**d**) Shows endovascular EVT cells within a trophoblast plug (red arrows) of a uterine blood vessel lined with vWF-positive endothelia. (**e**) Shows the same trophoblast plug from an adjacent serial section stained for ITGB1. A higher magnification of the inset, shown in (**f**), reveals that endovascular EVT cells (red arrows) express ITGB1 on their cell surfaces.

and lack villous trophoblast markers (ITGA6, cadherin 1, pregnancy specific beta-1-glycoprotein 1). These protein expression profiles are consistent with EVT cells present in the basal plate and lumen of the uterus during pregnancy (Moser *et al.*, 2010, 2015), as well as observations during EVT differentiation *in vitro* and *in vivo* (Damsky *et al.*, 1994; Zhou *et al.*, 1997). The use of anti-HLA-G appears to narrowly target *TRIC* for capture of cells with an EVT phenotype. Therefore, it remains feasible that other placental cells with a different lineage or phenotype also populate endocervical specimens, but are not captured by *TRIC*.

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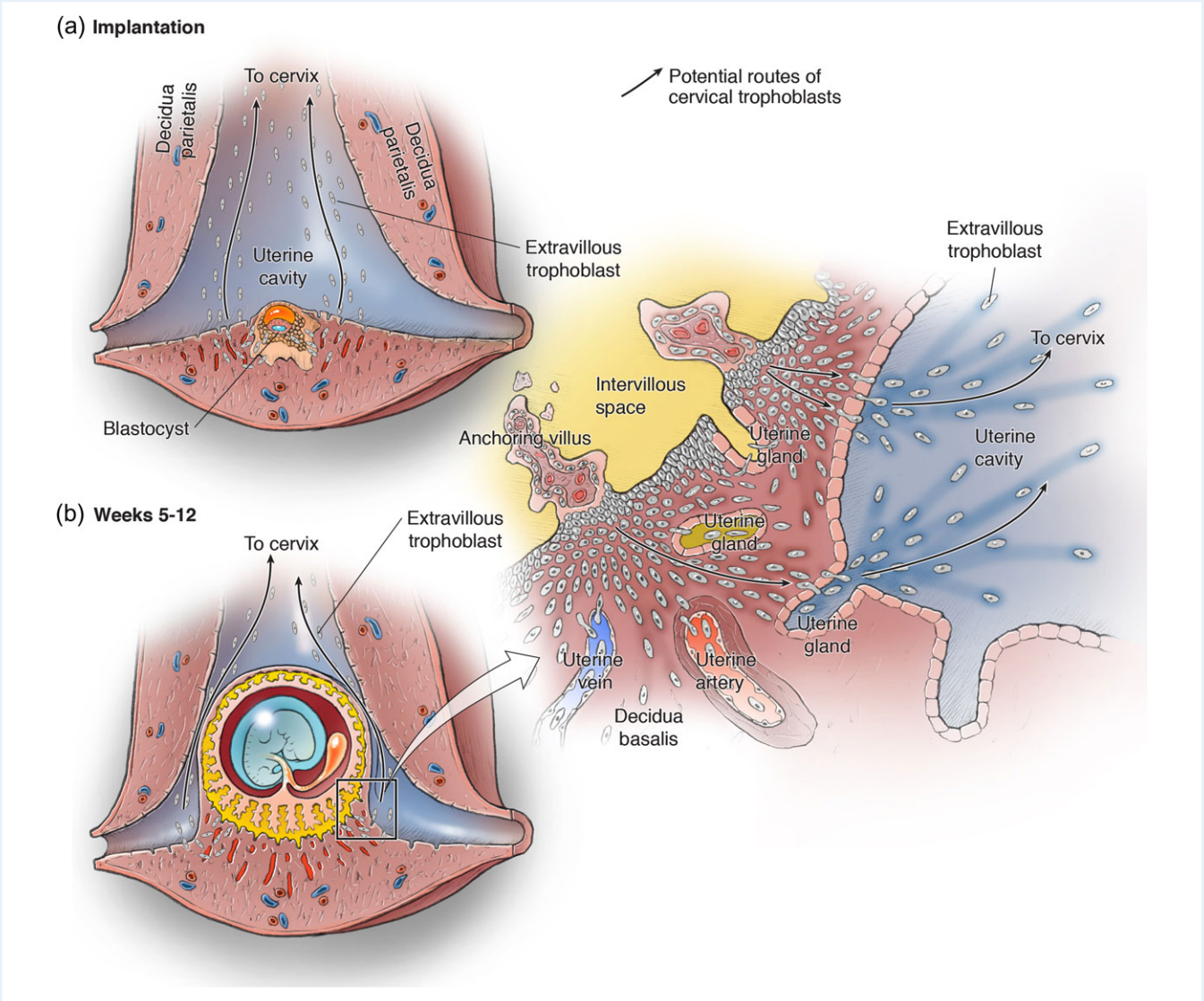

Figure 4 Origin of cervical trophoblast cells during placental development. The developing conceptus is shown within the uterus at the implantation site (**a**) and later during the placentation period of weeks 5–12 of gestation (**b**). EVT cells originate from trophoblast cell columns at the base of the anchoring villi, and follow the interstitial, endovascular, and endoglandular invasion routes. The inset in (**b**) is expanded to the right, showing the transitional zone of the decidua basalis at the margin of the placenta. As demonstrated in Figs 1 and 2, interstitial EVT cells can invade and replace the uterine epithelium from the basal side, and then enter the uterine cavity. In the decidua basalis, endoglandular EVT cells invade and reach the lumen of UGs. We speculate that, at the lateral margin of the placenta, they are transported together with the glandular secretions into the uterine cavity. Once EVT cells have reached the uterine cavity, they could migrate towards the cervix, or be carried there by the uterine secretion products (arrows).

Pregnancy pathologies and *TRIC*

For *TRIC* to be a truly robust approach to investigate fetal genetics, human placentation and associated pathologies, it is critical that adequate numbers of cells are available from all pregnancies regardless of their GA or the presence of pathology. It has been suggested that placental HLA-G expression is reduced by pre-eclampsia (Yie *et al.*, 2004; Zhu *et al.*, 2012), which could compromise the recovery of EVT cells by *TRIC*: these studies found reduced expression with GA, and examined the effect of pre-eclampsia at term. HLA-G protein levels were significantly reduced in placentas of women with pre-eclampsia, but maintained about half the expression level of healthy pregnancies (Zhu *et al.*, 2012). Because HLA-G is expressed with the onset of EVT differentiation (McMaster *et al.*, 1995), fewer EVT cells are present in fetal membrane or decidua of pre-eclamptic than normal pregnancies, but equivalent amounts of HLA-G mRNA are found when normalized to trophoblast content (Colbern *et al.*, 1994). Cytotrophoblast cells isolated from second trimester placentas that are induced to differentiate to EVT on Matrigel upregulate HLA-G mRNA and protein, but levels are significantly reduced in cultures of cells from pre-eclamptic pregnancies (Lim *et al.*, 1997). While disease

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might reduce HLA-G expression by EVT cells, there could remain sufficient amounts for immunomagnetic isolation by *TRIC*. A significant 4–5-fold reduction in the relative number of HLA-G positive cells present in endocervical specimens was observed in women with an ectopic or blighted ovum pregnancy (Imudia *et al.*, 2009). However, a survey of 224 ongoing pregnancies demonstrated that the number of cells obtained by *TRIC* is reduced by ~50% ($P < 0.05$) prior to an early pregnancy loss, but provides sufficient numbers (hundreds) of EVT cells for most downstream analyses (Fritz *et al.*, 2015b). The Fritz *et al.* (2015b) study further revealed no significant reduction of EVT cells isolated by *TRIC* in women who later developed pre-eclampsia or fetal growth restriction (FGR), and regression analysis showed no change in the number of EVT cells in specimens from women with an elevated BMI. *TRIC* is available 4–5 weeks earlier than other modalities for prenatal testing, at 5 weeks GA, and cell yield is unaffected by GA until 20 weeks, when it abruptly declines (Fritz *et al.*, 2015b). Thus, *TRIC* can be useful for investigation of early placentation and prenatal testing during a period of development when there is currently a significant gap in knowledge.

Utility of *TRIC* for Placental Research

The availability of EVT cells during ongoing pregnancies as a result of the *TRIC* methodology is unique and offers a powerful research tool that not only enables the use of cellular and molecular evaluation in the first trimester, but also provides information within the context of subsequent pregnancy outcomes. The literature provides strong support for the hypothesis that EVT cells are required for a normal term pregnancy by facilitating key physiological changes during placentation.

Trophoblast development and routes of invasion

The trophoblast lineage initially emerges during preimplantation development, and produces the trophectoderm of the blastocyst (Wiley, 1988; Collins and Fleming, 1995). As the first epithelium to form during human development, trophoblast cells retain expression of cytokeratin 7 even after differentiation to a non-epithelial EVT phenotype (Gauster *et al.*, 2013). Trophoblast cells in the blastocyst initially differentiate by fusion to produce a syncytiotrophoblast during implantation, which can remove the uterine epithelium and invade the stroma (Fig. 4a). Later, the chorionic villi form (Fig. 4b) and the villous trophoblast generate a stratified epithelium composed of mononuclear cytotrophoblast underlying a syncytiotrophoblast that directly contacts maternal fluids in the intervillous space. At the basal plate (Fig. 4b, expanded view of inset), proliferating cytotrophoblast cells at the bases of anchoring villi generate columns where they contact the decidua. Distally, the cytotrophoblast cells differentiate into EVT cells that invade the decidua and advance into uterine blood vessels and glands. Endovascular EVT cells invade and open the uterine veins (He *et al.*, 2017; Moser and Huppertz, 2017; Moser *et al.*, 2017; Windsperger *et al.*, 2017), as well as remodel the spiral arteries by disrupting the smooth muscle and tunica media, replacing the endothelia, and expressing endothelial proteins (Zhou *et al.*, 1997). The remodeled arteries form wide, low-resistance conduits of maternal blood that can more efficiently perfuse the chorionic villi to support the growing conceptus.

Trophoblast invasion in pregnancy pathologies

Incomplete or failed remodeling of the spiral arteries is hypothesized to be associated with dysregulated placental perfusion, in which perfusion of the placenta might be irregular, too fast or inadequate (Chaddha *et al.*, 2004; Burton *et al.*, 2009). When severe, dysregulated placental perfusion may even cause an early pregnancy loss. Pregnancies that endure could have an inadequately perfused placenta, and the associated pathology of FGR, or the hypertensive disorder pre-eclampsia (Burton and Jauniaux, 2004). There is also evidence that preterm labor can be associated with dysregulated placental perfusion (Papageorghiou *et al.*, 2004; Hossain and Paidas, 2007). The incidence of early pregnancy loss approaches 15% (Larsen *et al.*, 2013). FGR occurs in 4–8% of all pregnancies in developed countries, often defined as below the fifth percentile of newborn weight (Savchev *et al.*, 2014). Pre-eclampsia has a frequency of ~5–7%, while the early onset form occurs at 0.5–1%. Pre-eclampsia is clinically defined by the presence of maternal hypertension after 20 weeks of pregnancy, occurring in a previously normotensive patient, and is mostly accompanied by proteinuria (Davey and MacGillivray, 1988; Tranquilli, 2013). Maternal death or serious long-term morbidity can occur, while abruption, preterm delivery and FGR contribute to perinatal fetal death and infant morbidity, with the associated costs of extensive neonatal hospitalization, and lifelong disabilities (Roberts *et al.*, 1998). Until recently, pre-eclampsia could be alleviated only by removal of the placenta (Roberts *et al.*, 1998), requiring delivery of the fetus and accounting for 15% of premature deliveries and the associated infant morbidity and mortality (Meis *et al.*, 1998). Intervention prior to 16 weeks GA with regimens of low-dose aspirin (Bujold *et al.*, 2014) or Pravastatin (Ramma and Ahmed, 2014) have shown promise in reducing the rate of pre-eclampsia in high-risk patients, providing a course of action when anticipated early.

Screening based on *TRIC* and other techniques

Evidence has been assembled using TRIC to demonstrate that while displaced from the environment of the placenta, EVT cells residing in the endocervical canal nevertheless provide information about pregnancy status prior to clinical diagnosis of malplacentation syndromes. In two studies (Fritz *et al.*, 2015a; Bolnick *et al.*, 2016b) a set of proteins (PP13, galectin 14, PAPP-A, PGF, alpha fetoprotein, FLT1, endoglin) associated with dysregulated placental perfusion were measured in EVT cells obtained by TRIC between 5 and 19 weeks. The results demonstrated significant differences in expression levels for all but one (PP13) of the seven proteins, as assessed by relative protein quantification, when comparing samples from control pregnancies to those that either ended with an early pregnancy loss (Fritz *et al.*, 2015a), or later developed pre-eclampsia and/or FGR (Bolnick *et al.*, 2016b). These relatively small pilot studies require expansion to screen a larger patient pool, and further suggest that EVT cells obtained by *TRIC* offer a valuable resource for exploratory studies

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using 'omics' approaches to identify new, more valuable biomarkers of obstetrical pathology, and for investigating the etiology of these diseases through pathway analysis.

TRIC, in providing access to EVT cells in ongoing pregnancies, is the first minimally invasive procedure that can potentially screen patients early in the first trimester to assess risk for dysregulated placental development and perfusion. If EVT molecular profiles prove to be capable of reliably identifying at-risk patients, novel interventions to prevent adverse pregnancy outcomes could more efficiently scrutinized by focusing on high-risk patients identified using TRIC; thereby reducing the number of subjects required to obtain definitive results. For example, a meta-analysis of 32 217 women participating in randomized trials to test the effect of various antiplatelet agents on pre-eclampsia showed a lack of benefit (Askie *et al.*, 2007). A limitation of such large studies is the infrequency of the adverse outcomes under study, thus, significantly reducing statistical power.

Recently, an algorithm was employed using first trimester biophysical and biochemical test results, as well as patient medical history, to identify women with a risk >1:100 for preterm pre-eclampsia in a double-blind, placebo-controlled trial (Rolnik *et al.*, 2017). Using this approach, 1620 women were selected for inclusion in the study after screening 26 941, and a significant reduction of preterm pre-eclampsia was obtained in the aspirin treatment group, compared to placebo. The predictive algorithm used in the Rolnik *et al.* (2017) study is not recommended currently by the US Preventive Services Task Force because screening based on clinical history appears to prevent pre-eclampsia in a reasonable fraction of cases without the additional costs of biophysical and biochemical testing (Roberts and Himes, 2017). In addition, other studies show that combining maternal characteristics, serum biomarkers and uterine artery pulsatility index between 11 and 13 + 6 weeks identifies only 60% of preterm pre-eclampsia, with a fixed false positive rate of 10% (Sonek *et al.*, 2017). Prediction of FGR (i.e. small for GA) is suboptimal as well (McCowan *et al.*, 2017). Because *TRIC* offers the ability to obtain information earlier in gestation that reflects embryonic/fetal status, it might be possible to use this approach to develop better intervention strategies.

Utility of *TRIC* for prenatal testing

Prediction of chromosomal and genetic disorders early in pregnancy provides vital information, allowing parents and caregivers to be proactive and prepare for the best possible outcome if congenital or inherited disorders are present. Down syndrome (trisomy 21) and other aneuploidies occur in over 0.2% of births worldwide, and are on the rise due to advanced women's age at the time of pregnancy. Single-gene mutations account for over 6000 diseases, 10% of all pediatric hospital admissions, and 20% of infant deaths. Currently, definitive diagnosis of fetal genetic traits requires invasive procedures; either CVS beginning at 9–10 weeks GA, or amniocentesis beginning at 12–14 weeks GA (Eisenberg and Wapner, 2002). Comprehensive interrogation of the fetal genome before 9 weeks is now unavailable. However, NIPT is useful as an early screen for common aneuploidies (Bianchi *et al.*, 2014). By 10 weeks GA, fetal DNA fragments comprise 4–10% of total cell-free DNA in maternal plasma, which can be distinguished from the maternal fraction by massively parallel sequencing and computational comparisons of single nucleotide polymorphisms (SNPs) or STRs (Wong and Lo, 2016). However, it is the opinion of professional medical organizations that a diagnostic test, which would require invasive procedures, should be offered to patients who have a positive cell-free DNA test result (ACOG/SMFM, 2015; Benn *et al.*, 2015; Gregg *et al.*, 2016).

TRIC is capable of providing highly purified EVT cells containing the intact and complete fetal genome for analysis, with minimal maternal DNA present that must be distinguished. As a result, fetal DNA analysis becomes more straightforward, simple and inexpensive than NIPT. While similar advantages have motivated investigators pursuing fetal cells in maternal blood, the cervix offers one or two orders of magnitude more cells and fetal DNA, making it a more practical approach for development of prenatal genetic tests. Early investigations demonstrated that fetal cells in the reproductive tract could provide clinically relevant genetic information (Canestrini and Testa, 1978), although the overwhelming background of maternal cells present made it impractical.

TRIC has been used successfully with FISH and PCR to unequivocally determine fetal sex (Bolnick *et al.*, 2014, 2016a). DNA fingerprinting by targeted next-generation sequencing of STRs and SNPs has now been successfully conducted with EVT cells obtained by *TRIC* (Jain *et al.*, 2016). There was a 100% correspondence between the SNP and STR haplotypes of fetal DNA obtained by *TRIC* and corresponding placental DNA in every specimen. These findings demonstrate single nucleotide resolution of fetal DNA, establishing the feasibility of safely analyzing single gene disorders as early as 5 weeks GA, earlier than other non-invasive or invasive methods. Future studies will test the limitations of *TRIC* for genetic testing and its potential suitability as a diagnostic method. Sequencing of loci amplified by multiplex PCR can be extremely robust, whereas whole exome and whole genome sequencing will require whole genome amplification, which can introduce bias and sequencing errors. However, as the methodologies improve, more downstream analytical approaches will become available with the limited amounts of DNA available through *TRIC*.

Conclusions

The existence of trophoblasts in the uterine cavity and endocervical canal has been known for many years; however, the technology is only now becoming available to access the information within those cells to probe the status of the placenta and pregnancy in real time. The *TRIC* procedure, in skilled hands, can isolate hundreds of fetal cells as early as 5 weeks of pregnancy from a simple pap smear. While single cell technology still lacks clinical precision, *TRIC*, as an intermediate solution, offers unique insights into human pregnancy and fetal development. It has advantages over cell-free fetal DNA in maternal serum because the isolated EVT cells contain the entire fetal genome for genotyping, while the problem of confined placental mosaicism limits the certainty of aneuploidy detection, as in NIPT and CVS. Although we do not yet understand the exact biological mechanisms that release EVT cells into the reproductive tract and the route that trophoblast cells take to the cervix, recent advances suggest that a new era of prenatal diagnosis lies ahead. There remains a paucity of information about differences that might exist between EVT cells in the placenta and those residing in the cervix that might

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color their utility as a barometer on pregnancy status and health, which needs to be resolved in future studies. Currently, measurements of disease biomarkers in maternal blood (e.g. PAPP-A, PP13, inhibin, hCG, PGF, FLT1) are under development to assess pregnancy. EVT cells accessible in the reproductive tract could potentially serve in that role at an earlier GA and with greater precision, since deficiencies of the EVT likely contribute to placental maldevelopment. With the use of *TRIC* for early prenatal testing now feasible, the future could hold opportunities to screen fetuses and mothers at risk in preparation for corrective interventions to reduce morbidity and mortality.

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Authors' role

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Conflict of interest

D.R.A. and S.D. have pending patents, receive payment for intellectual property that has been licensed by Wayne State University to PerkinElmer, Inc., and are principals in Advanced Reproductive Testing, LLC.

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