

HTR-8/SVneo cell line contains a mixed population of cells



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ABSTRACT

Introduction: The placenta, a transient organ in humans, is essential for pregnancy maintenance and fetal development. Trophoblast and stromal cells are the main cell types present in human placenta. Trophoblast cells are derivatives of the trophoblast layer and fulfill the endocrine, exchange, invasion and implantation processes of the placenta, whereas stromal cells are of extraembryonic mesenchymal origin and are important for villous formation and maintenance. Different cell lines were developed to study trophoblast functions including BeWo, JEG-3 and JAR from chorioncarcinoma while HTR-8/SVneo was developed using first trimester extravillous trophoblast infected with simian virus 40 large T antigen (SV40). These cell lines are largely used to study trophoblast functions including cell fusion, migration and invasion. Therefore, the purity of each cell lines is crucial in order to be able to use them as a model recapitulating trophoblast cells.

Methods: HTR-8/SVneo, BeWo, JEG-3 and JAR were analyzed for epithelial and mesenchymal markers using immunofluorescence, real time PCR and Western blot.

Results: Our results showed that HTR-8/SVneo cell line contains two populations of cells suggesting the presence of trophoblast and stromal/mesenchymal cells. While all cells in BeWo, JEG-3 and Jar are positive for the trophoblast/epithelial marker CK7, HTR-8/SVneo cells contained few clusters of CK7 positive cells. Interestingly, vimentin expression was detected in a subset of HTR-8/SVneo cells and was completely absent from all other tested placental cell lines.

Discussion: Our results unveil the presence of a heterogeneous population of trophoblast and stromal cells within HTR-8/SVneo cell line. This mixed population of cells should be taken into consideration when using this cell line to study trophoblast functions.

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1. Introduction

The human placenta plays a major role in the maintenance of pregnancy and in fetal development. A normal placental development involves two pathways of trophoblast differentiation that lead to the formation of two distinct phenotypes: extravillous and villous cytotrophoblasts [1]. The extravillous cytotrophoblasts (evCTB) are responsible for endometrial invasion and implantation while villous cytotrophoblasts (vCTB) are required for nutrient and waste exchange between the mother and fetus. This latter function is associated with the differentiation of specific structure: the

syncytiotrophoblast (STB) which arises from the differentiation and fusion of the relatively undifferentiated mitotically active villous cytotrophoblast [2]. Therefore, alterations in these differentiation pathways have a major impact on placental maintenance and integrity and consequently its role as barrier and exchange organ. Moreover, abnormal syncytial fusion seems to be implicated in the development of many pregnancy-related diseases such as Down syndrome and pre-eclampsia [3]. Consequently, different systems were developed to study these differentiation processes including cell and tissue explants culture or *in vivo* models [4–6]. However, each model has its advantages and disadvantages.

Among the cell culture models, freshly isolated cytotrophoblast are largely used to study trophoblast differentiation and invasion/migration [7–10]. When freshly isolated cytotrophoblast are cultured in 10% FBS, they spontaneously fuse and form syncytiotrophoblast [9,11,12]. However, the major drawback of freshly isolated cytotrophoblast is related to their lifespan in culture (maximum 7 days) and to their inability to divide [7,9]. Therefore,

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different placental cell lines were developed and many of them are derived from choriocarcinoma, a trophoblastic malignant cancer.

In 1959, Hertz et al. was able to isolate trophoblast cells from an autopsy of a cerebral metastasis of a choriocarcinoma, which he then transplanted to the cheek pouch of a hamster over a period of 8 years [13]. Then, the BeWo cell line was established once cells from the tumors were removed from the cheek pouches and cocultured with decidua tissues [14]. BeWo cell line became the most widely used cell culture model to mimic vCTB. BeWo cells are able to fuse and form STB [15,16], regulate syncytin 1 and 2 [17] and secrete hormones such as hCG, hPL, progesterone and estradiol [18]. Besides, JEG-3 cells were derived by serial cloning of BeWo cells into the hamster cheek pouch. JEG-3 cells also release hCG, hPL and progesterone [14]. JAR cell line was also derived from a trophoblastic tumor of the placenta [19]. In parallel, different cell lines were developed using first trimester evCTB infected with different retroviral vectors to mimic evCTB.

The first evCTB cell line was developed by Graham et al. [20] and named HTR-8/SVneo. It was generated using freshly isolated evCTB from first trimester placenta and transfected with a plasmid containing the simian virus 40 large T antigen (SV40). Thereafter, different evCTB cell lines were developed using first trimester placenta such as TEV-1, ACH-3P, SGHPL-5 and HIPEC65 [21–24]. Among all these evCTB cell lines, HTR-8/SVneo remains the most commonly used to study evCTB invasion, proliferation and regulation [25–27]. However, this cell line was generated 3 decades ago and characterized as epithelial cell using an antibody that recognizes CK8 and 18, and as trophoblast using the level of secreted hCG [20]. It should be noted that beta-hCG was only detected in transfected HTR-8/SVneo and not in parental HTR-8 cells [20]. Furthermore, Chen *et al* showed that the expression of CK7 and E-cadherin is silenced in HTR-8/SVneo while a high expression of vimentin was observed [28]. Moreover, Gierman *et al.* showed, based on toll-like receptors (TLR) profiling, that SGHPL-5 most closely resembled primary extravillous trophoblast when compared to HTR-8/SVneo [29]. All these observations prompted us to investigate the purity and the phenotype of HTR-8/SVneo.

2. Materials and methods

2.1. Cell culture

BeWo (ATCC, CCL-98), JEG-3 (ATCC, HTB-36), JAR (ATCC, HTB144) and HTR-8/SVneo cell lines were a gift from Dr. Cathy Vaillancourt (INRS, Quebec, Canada). BeWo and HTR-8/SVneo cells were cultured in F-12K medium and DMEM (Sigma-Aldrich) respectively while JEG-3 and JAR were cultured in MEM (Sigma-Aldrich). All culture media were supplemented with 10% heat inactivated FBS (Gibco) and Penicillin/Streptomycin (100 U/ml, Lonza). Cells were cultured in T75 cm² flask at 37 °C, 5% CO₂ and humidified atmosphere and subcultured using 0.25% trypsin-EDTA.

2.2. Immunofluorescence and confocal microscopy

2.2.1. Antibodies and reagents

Antibodies from the indicated manufacturers used in this study were as follows: mouse E-cadherin (cat: ab1416, Abcam Inc., MA), rabbit e-cadherin (cat: 24E10, Cell Signaling Technology, MA), rabbit Vimentin (cat: sc7557, Santa Cruz Biotechnology, CA), mouse HLA-G (cat: ab7758, Abcam Inc., MA) and mouse CK7 (cat: ab9021, Abcam Inc., MA). Secondary antibodies used are the following: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit (Invitrogen, CA). All antibodies were used at 1/100 dilution. Fluoro-gel II with DAPI was purchased from EMS (Electron

Microscopy Sciences, PA).

2.2.2. Immunofluorescent staining

Immunofluorescent studies were done as previously described [4]. Briefly, cells were cultured on coverslips (15 mm) in 12-well plates until they reach 70–80% confluency. Adherent cells were fixed in 4% paraformaldehyde (PFA) for 15 min, followed by permeabilization with 0.05% Triton X-100 for 5 min. Non-specific sites were blocked by incubation in 0.1% BSA in PBS for 60 min. For HLA-G staining, cells were incubated directly with anti-HLA-G without fixation as per manufacturer's instructions. Cells were incubated overnight at 4 °C with the specified primary antibodies diluted in the blocking buffer. Thereafter, cells were washed and incubated with either Alexa Fluor 488 or 568 conjugated goat anti-mouse and goat anti-rabbit IgG in blocking buffer for 60 min at room temperature. Finally, coverslips were mounted using the anti-fade reagent Fluoro-gel II with DAPI. Confocal microscopic analyses were performed using Zeiss LSM 710 confocal microscope and images were acquired and analyzed using the ZEN 2012 image software.

2.3. RNA extraction, reverse transcriptase and real-time PCR

Cells were cultured in 6-well plates and RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and converted into cDNA using the RevertAid First strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions.

Gene expression was analyzed and quantified by real-time PCR using specific primers (Table 1). The PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 58 °C for 30 s and 72 °C for 1 min. Fold changes in gene expression were calculated according to the Relative quantitation method ($\Delta\Delta CT$) using GAPDH as reference gene as previously described [30]. Each sample was analyzed in duplicate from three different experiments.

2.4. Western blot analysis

BeWo, JEG-3, JAR and HTR-8/SVneo cells were lysed using RIPA buffer containing 50 mM Tris-HCl, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxyolate, 100 mM EDTA, 1% Tergitol (NP40), 1 mM PMSF and protease and phosphatase inhibitors (1 tablet of each in 10 ml buffer, Roche). Cells were incubated in RIPA buffer for 1 h on ice, vortexed and then centrifuge at 4 °C for 15 min at 14,000 rpm. The supernatant was collected and protein concentrations were quantified using Bradford Protein Assay. For immunoblotting, 25–50 μ g of proteins were loaded onto 10% polyacrylamide gel and allowed to migrate at 100 V for 2–3 h and then transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA) for 2 h. Membranes were blocked in 5% low fat milk and incubated at 4 °C overnight with primary antibodies as follows: rabbit anti-E-cadherin (1/1000 dilution, cat: 24E10, Cell Signaling Technology, MA), rabbit anti-Vimentin (1/1000 dilution, cat:

Table 1
Primer sequences and annealing temperature used in the real-time PCR.

Gene	Sequence	Annealing T (°C)
GAPDH	F: 5'- TGGTGCTCAGTGTAGCCCAG -3' R: 3'-GGACCTGACCTGCCGTCTAG-5'	58 °C
CK7	F: 5'- GGTTCCTGGAGCAGCAGAAC -3' R: 5'- AAGTCCTCCACCACATCCTG -3'	58 °C
Vimentin	F: 5'- ACCAACGACAAAGCCCGCGT-3' R: 5'- CAGAGACGCATTGTCAACATCCTGT-3'	58 °C
EpCAM	F: 5'-CCATGTGCTGTGTGTAAC-3' R: 5'-ACGCGTGTGATCTCCTTCT-3'	58 °C

sc7557, Santa Cruz Biotechnology, CA) and mouse anti-CK7 (1/1000 dilution, cat: ab9021, Abcam Inc., MA) and mouse anti-GAPDH (1/4000 dilution, cat: NB300-221, Novus Biologicals, USA). After incubation, membranes were washed and incubated with HRP-conjugated secondary antibodies (Goat anti-mouse, sc2031 and Goat anti-rabbit, sc2030; Santa Cruz Biotechnology, CA at 1/2000 dilution). Proteins were detected using Chemiluminescence system (Roche) and visualized using autoradiography. Densitometry analysis of band intensity was digitized and analyzed using ImageJ software (National Institute of Health, NIH).

2.5. Statistical analysis

Data are expressed as the mean \pm S.D and analyzed with *t*-test at a *p* value < 0.05 using GraphPad Prism 5 analysis software.

3. Results

3.1. HTR-8/SVneo cell line contains two populations of cells

Multiple cell lines are used to study placental development and physiology. Among these cell lines, BeWo, JEG-3 and JAR are derived from chorioncarcinoma [14,19,31] while HTR-8/SVneo is derived from normal first trimester placenta [20]. These cell lines are widely used to study multiple physiological processes including trophoblast differentiation, adhesion, migration and implantation. Interestingly, under normal culture conditions, BeWo, JEG-3 and JAR cells showed homogenous epithelial population (cell-cell adhesion) while HTR-8/SVneo cells showed heterogeneous population containing epithelial and mesenchymal-like (migratory

phenotype with minimal cell-cell adhesion) cells (Fig. 1A). In order to investigate this observation, all four cell lines were stained with CK7, a well-known epithelial marker for trophoblast cells. Interestingly, CK7 staining showed positive for most BeWo, JEG-3 and JAR cells while just few cells were positive in HTR-8/SVneo (Fig. 1B). These results showed the presence of two cell populations in HTR-8/SVneo, CK7 positive and CK7 negative cells.

3.2. HTR-8/SVneo cell line contains epithelial and mesenchymal cells

In order to identify the type of these two populations, epithelial (CK7 and E-cadherin) and mesenchymal (Vimentin) markers were used. Fig. 2 clearly shows the presence of two distinct populations of cells in HTR-8/SVneo cell line; the CK7 or E-cad positive/vimentin negative population (Fig. 2A, B and C) and CK7 or E-Cad negative/Vimentin positive (Fig. 2A and B). Fig. 2 revealed the presence of different clusters of these epithelial and mesenchymal populations. A higher magnification image clearly showed the presence of these two distinct populations (Supplemental Fig. 1). Furthermore, *HLA-G* gene is largely used as extravillous trophoblast marker [32–34] and HTR-8/SVneo were isolated and purified as extravillous trophoblast from first trimester placenta [20]. Therefore, we used anti-*HLA-G* to detect extravillous trophoblast in HTR-8/SVneo cells. As previously shown for CK7 (Fig. 1), few cells were positive for *HLA-G* (Supplemental Fig. 2). A double staining of CK7/*HLA-G* or vimentin/*HLA-G* was not possible due to two different staining protocols. *HLA-G* required staining of live cells without fixation while CK7/vimentin required fixation followed by staining (data not shown).

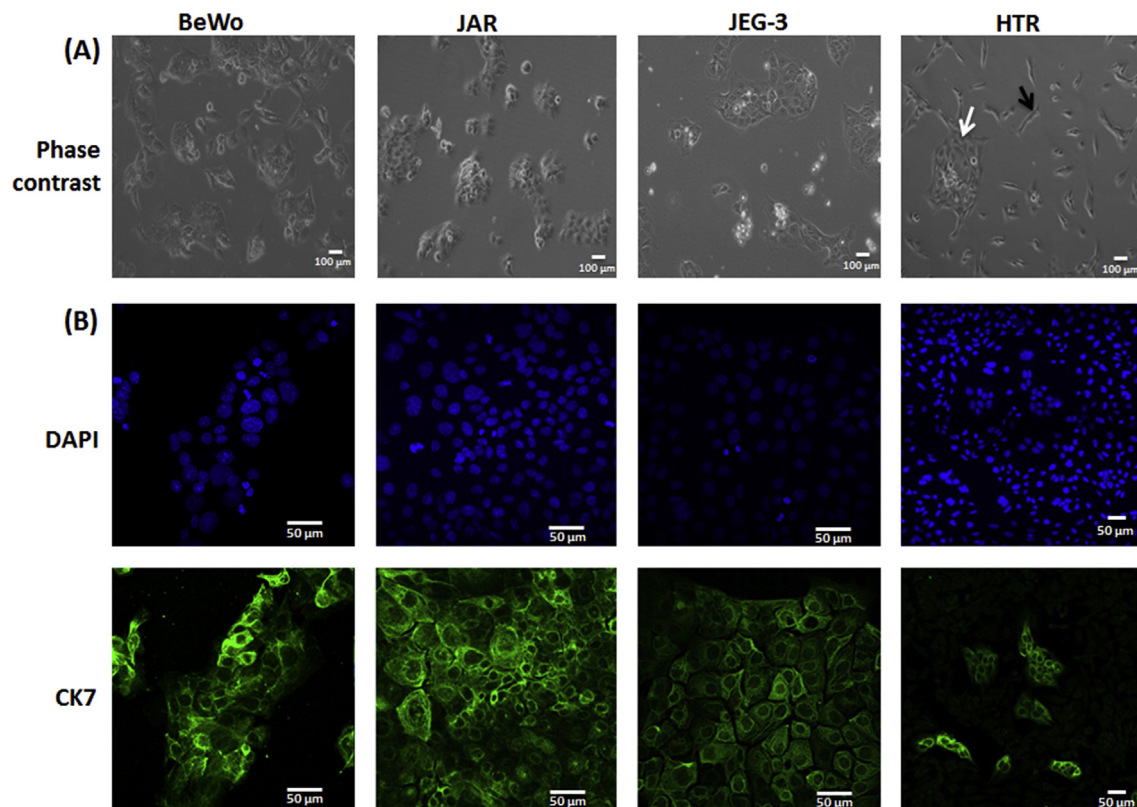


Fig. 1. HTR-8/SVneo cell line contains two populations of cells compared to BeWo, JAR or JEG-3.

A) Cells were cultured in T75 flask and phase contrast images were taken. HTR-8/SVneo showed the presence of epithelial-like population (white arrow) and mesenchymal-like population (black arrow) while all other tested cell lines were homogenous. **(B)** Cells were stained with anti-CK7 (green) and DAPI (blue). Data are representative of at least 3 different experiments. Scale bar = 50 and 100 μm.

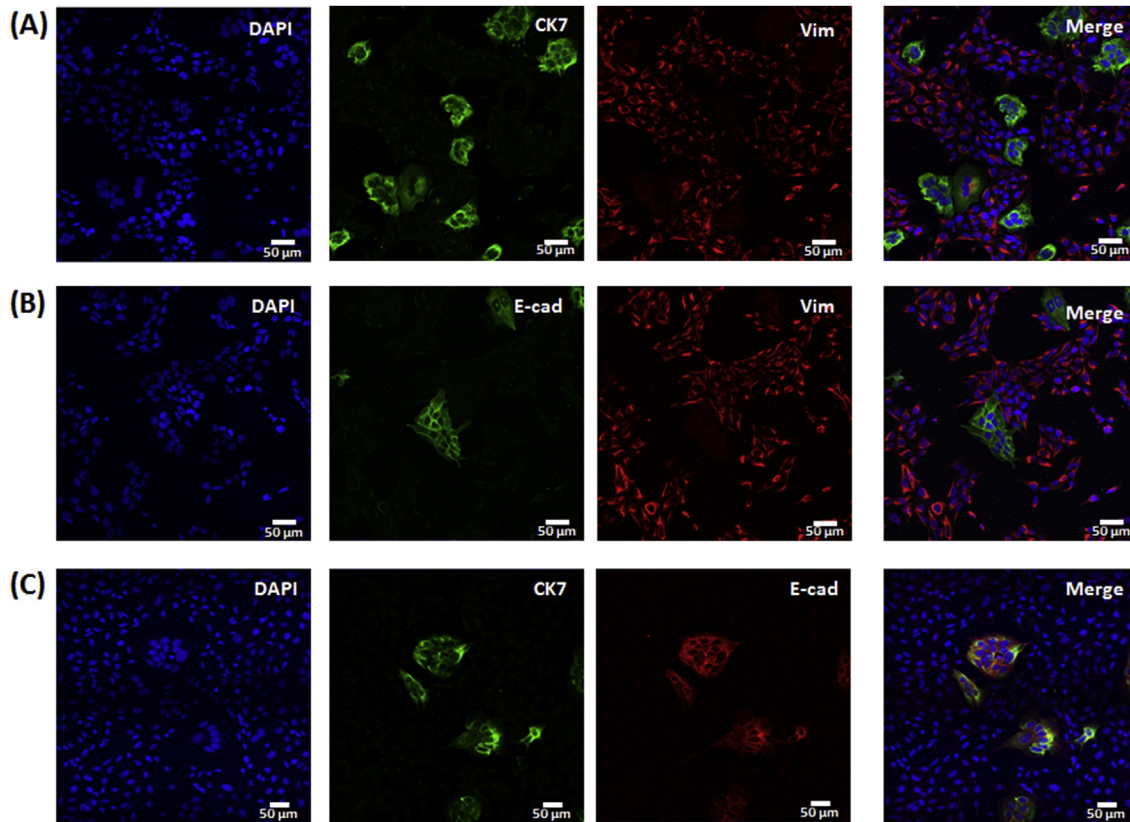


Fig. 2. HTR-8/SVneo contains CK7⁺/vimentin⁻ and CK7⁻/vimentin⁺ cells.

HTR-8/SVneo cells were cultured on coverslips and stained with anti-CK7, E-cad or vimentin and DAPI. (A) CK7 positive and vimentin negative HTR8-SVneo cells. (B) E-cadherin positive and vimentin negative HTR8-SVneo cells. (C) CK7 and E-cadherin positive HTR8-SVneo cells. Data are representative of at least 3 different experiments. Scale bar = 50 µm.

3.3. BeWo, JEG-3 and JAR are purely epithelial cells

In order to validate our observation, we used the other known cell lines to confirm their epithelial phenotype without the presence of any mesenchymal phenotype. Therefore, BeWo, JEG-3 and JAR cells were stained with CK7, E-cadherin and vimentin to confirm their epithelial/trophoblast phenotype. Fig. 3 shows that all BeWo, JEG3 and JAR cells are positive for E-cadherin and CK7 with no staining for vimentin observed confirming their pure epithelial phenotype.

3.4. HTR-8/SVneo cells express high levels of vimentin and traces of CK7 and E-Cadherin or EpCAM

To further confirm the presence of mesenchymal and epithelial cells in HTR-8/SVneo, gene expression profile was evaluated using specific markers by real-time PCR (Fig. 4) and Western blot (Fig. 5) analysis. Fig. 4 shows the mRNA of vimentin, CK7 and EpCam in all 4 cell lines using real time PCR. Interestingly, the expression of vimentin was only detected in HTR-8/SVneo cells by real time PCR (Fig. 4) while CK7 and Epcam showed a modest expression in HTR-8/SVneo compared to BeWo, JEG-3 and JAR. Furthermore, the same results were obtained by Western blot showing a high expression of vimentin and barely a detectable levels of CK7 and E-cadherin in HTR-8/SVneo (Fig. 5). All these data confirm our immunofluorescence observation validating the presence of two populations in HTR-8/SVneo cell line, both the epithelial and mesenchymal cell populations.

4. Discussion

This study shows that HTR-8/SVneo cell line contains heterogeneous population of cells with different phenotypes. Some cells express CK7 and E-cadherin while others express vimentin. It should be noted that keratins and e-cadherin are largely used as markers of epithelial cells [35,36] while vimentin is used as mesenchymal marker [37]. Our immunofluorescence results clearly showed the presence of these two populations in HTR-8/SVneo cell line, which was further confirmed by real time PCR and western blot analyses.

HTR-8/SVneo cell line was isolated from first trimester placenta and validated as trophoblast cell line with extended lifespan [20]. It was characterized as an epithelial/trophoblast cell line based on the expression of CK8/18 and low level of hCG secretion [20]. Currently, CK7 is the gold standard marker used to identify all different types of trophoblast cells [30,38]. Our results showed that not all HTR-8/SVneo cells express CK7, suggesting that some cells may not be trophoblasts and hence this cell line may be containing a mixed population of cells. It has been previously proven that CK8/CK18 are not reliable markers for neither epithelial cells nor trophoblast cells since they were also detected in mesenchymal cells of the human first trimester placenta [39,40]. Moreover, HLA-G is a typical marker of extravillous trophoblasts [41]. In this study, we showed that some clusters of cells express HLA-G while most do not. This finding is in accordance with other studies showing that HTR-8/SVneo cells slightly express HLA-G or not at all [42] and have less in common with the primary extravillous trophoblast than JEG-3 cells [43], which further underlines the possibility of HTR-8/SVneo being a heterogeneous cell line of trophoblast and

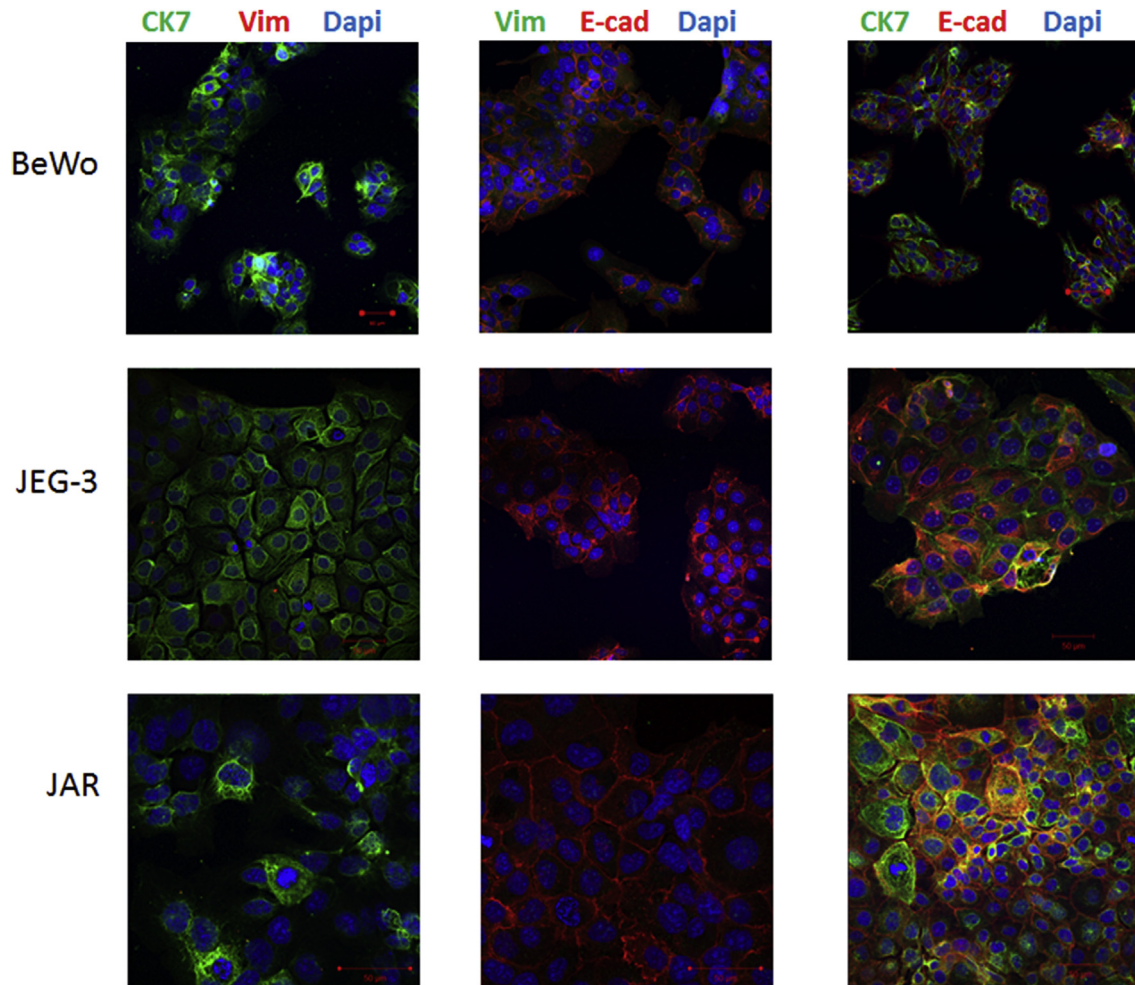


Fig. 3. BeWo, JEG-3 and JAR cells are CK7/E-cadherin positive and vimentin negative. Cells were cultured on coverslips and stained with anti- CK7, E-cad or vimentin and DAPI. Data are representative of at least 3 different experiments. Scale bar = 50 μ m.

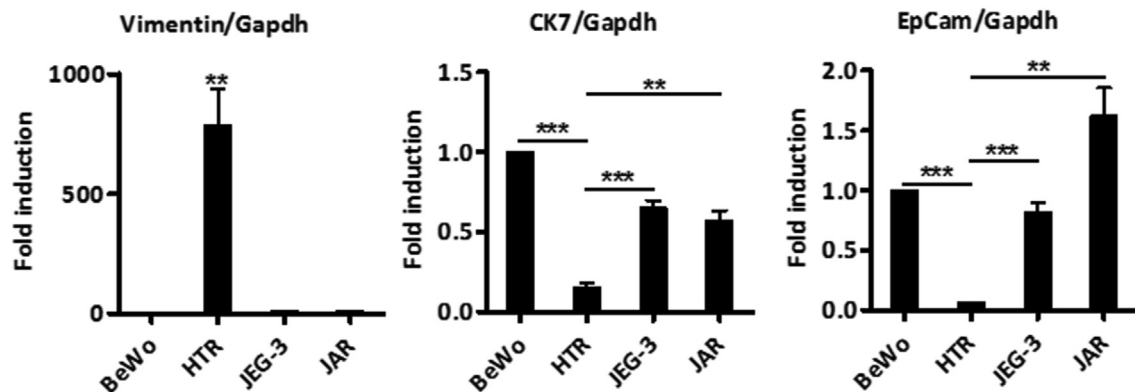


Fig. 4. Real time PCR showing the expression of Vimentin, CK7 and EpCAM in BeWo, HTR-8/SVneo, JEG-3 and JAR. Cells were cultured in 6-well plates and RNA expression was evaluated by real-time PCR using specific primers listed in Table 1. Data are presented as fold change compared to BeWo cells from 3 different experiments. (**p < 0.01 and ***p < 0.001; t-test).

mesenchymal cells. This mixed population might have originated during the purification and generation of HTR-8/SVneo [20]. This hypothesis is plausible because the authors did not test for vimentin expression in their culture while our results and others showed clearly a vimentin expression in these cells [28]. Notably, primary culture trophoblasts are characterized by negative

vimentin expression [39,44,45]. Moreover, CK8/18 was used as epithelial marker which is also expressed in placental stromal cells as discussed above.

Another plausible hypothesis to explain the observed phenomenon in HTR-8/SVneo is the epithelial-to-mesenchymal transition (EMT) phenomenon. A recent study showed that the

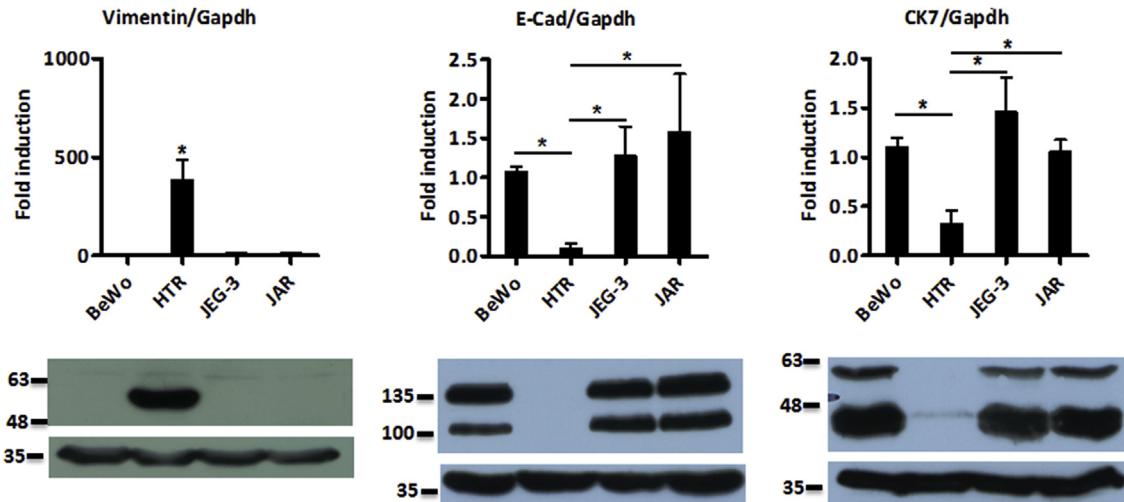


Fig. 5. Western blot analysis showing the expression of Vimentin, CK7 and E-Cadherin in BeWo, HTR-8/SVneo, JEG-3 and JAR.

Cells were cultured in 6-well plates and total protein was extracted in RIPA buffer. 25–50 μ g of total proteins was subjected to 10% SDS-PAGE. Protein expression was evaluated by western blot using specific antibodies as described in materials and methods. GAPDH was used as loading control. Densitometry analysis of protein levels after normalization with GAPDH is also provided. Data are representative of at least 3 different experiments.

transformation of cytotrophoblast to extravillous trophoblast is a unique form of EMT [46]. Using microarray analysis, authors showed that evCTB cells generated at the tips of the anchoring villi loose epithelial markers and acquire mesenchymal markers when they start to invade maternal endometrium and spiral arteries. Some of the downregulated epithelial markers included E-cadherin and occludin while upregulated mesenchymal markers included vimentin, fibronectin, MMP2 and MMP9 [46]. While HTR-8/SVneo might have been originally trophoblastic in nature, it might have been subjected to EMT upon transformation with SV40 or in culture. Some primary epithelial cultures have been shown to express mesenchymal markers when they were subjected to extension of their lifespans [47]. Interestingly, HTR-8/SVneo cells express N-cadherin [48] and vimentin, both known as EMT markers [49]. Moreover, some contradictory results were reported about the expression of CK7 in evCTB. While Muhlhauser et al. reported that extravillous trophoblasts highly express CK7 which is then decreased during migration and invasion until its expression completely disappears [50], another study showed that HLA-G positive trophoblast cells sorted using FACS are 95% positive for CK7 and designated as evCTB [46]. Moreover, it has been shown that the expression of CK7 and E-cadherin in HTR-8/SVneo is shut down due to hypermethylation of their respective promoters and that this inhibition was relieved when an inhibitor of DNA methyltransferase is used [28].

Noteworthy, our results showed 2 populations of cells in HTR-8/SVneo as regards to CK7 expression. One population highly expressed CK7 while the other lacked CK7 expression. If we accept the hypothesis that evCTB highly express CK7 at the tip of the anchoring villi and start to decrease its expression while migrating and invading the maternal endometrium due to an EMT process as described above, these two populations of cells in HTR-8/SVneo might be explained by a normal ongoing EMT process. To test and validate this hypothesis, we are currently subcloning HTR-8/SVneo cells and single clones are being selected. These subclones will be tested for CK7 and vimentin expression early after selection and will be then monitored for both markers expression in later passages. Our preliminary data show that some clones express only CK7 or vimentin in early passages (2–3 passages) and we are currently maintaining these clones in culture to screen them in later passages for these markers to validate the occurrence or not of

EMT in normal culture conditions.

In conclusion, our results raised the question about using HTR-8/SVneo as model for evCTB to study placental physiology. We clearly showed that this cell line contains 2 populations of cells and therefore should be used with precautions as it contains both epithelial and mesenchymal-like cells.

Author contributions

W.A.K., and G.D. were responsible for the conception and design of the study, analysis and interpretation of data and drafting the article. J.B. and O.H were responsible for experimental design and data acquisition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.placenta.2016.12.007>.

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