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RESEARCH ARTICLE

Isolation and characterization of trophoblasts from enzymatic explants of human term placenta

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Abstract In the present study, we describe a new method of isolation and culture of human villous and extravillous trophoblasts from term placenta. The cultivation of trypsinized placental villous tissue explants, followed by the isolation of cells from outgrowth islets allows for obtaining a cytotrophoblast subpopulation that is free from contamination by other cell types. Compared to other methods, our protocol is mild, simple and effective, does not request costly reagents and provides isolation of the mononuclear cytotrophoblast cell populations free from contamination by other types of placental cells. The isolated cells proliferated and formed a pleomorphic monolayer, where cells fused into a small number of binuclear or polynuclear syncytiotrophoblasts. Isolated cytotrophoblast cells expressed the specific epithelial intermediate filament cytokeratin 7 (CK7), the epithelium-specific cell–cell adhesion molecule E-cadherin and were CD9-, CD45- and vimentin-negative. Cyto- and syncytiotrophoblasts obtained by this method can be used as a model or tool for the fundamental research of differentiation and function of human placental cells, and can provide a new understanding of drug distribution in placenta. Their combination with other in vitro cell models can be useful for studying a variety of other aspects concerning placental functions, which will provide new knowledge for understanding immunology, endocrinology and development of placenta.

Keywords Cytotrophoblast · Cell culture · Cell isolation method · Human term placenta · Villous enzymatic explants

Introduction

Trophoblast cell cultures are considered the most promising model for studying mechanisms and the control of human placental trans-syncytiotrophoblast transfer, drug distribution and the process of trophoblast growth and differentiation [1]. This model allows for studying the role of cells as part of the process of the transfer and utilization of tested products during transport through placenta [2]. Commercially available trophoblast cell lines BeWo, JEG-3 or JAR derived from human choriocarcinoma have proven to be extremely valuable for the study of the cellular, molecular and endocrine aspects of human trophoblasts' function [3, 4]. However, they do not exactly reflect the normal behaviour of the cyto- and syncytiotrophoblast cells. The expression and function of several enzymes and transporters in these transformed cells are altered, thereby affecting the extrapolation of data to the in vivo condition.

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The study of human trophoblast function in vitro has been hampered by difficulties in the isolation and cultivation of primary human trophoblasts, and by contamination of culture with other cell types. The digestion of placental tissue results in obtaining a mixture of various cell types, including villous trophoblasts, extravillous trophoblasts, placental fibroblasts, stromal and endothelial cells, as well as a large amount of blood cells and debris. The use of hESC (human embryonic stem cells) to derive trophoblasts remains problematic, due to the heterogeneity of the obtained 'trophoblast' population [5, 6].

Nowadays, the isolation of villous trophoblast cells from placental tissue is considered a simple and routine process. Nevertheless, there are differences to the populations of trophoblast cells obtained by various protocols. Different enzymes (trypsin, collagenase, DNAse type IV, dispase, etc.) and various conditions are used for cell isolation from placenta [2, 7–11]. The traditional isolation procedure of villous trophoblasts is based on the digestion of placental villous pieces in trypsin and DNAse, followed by further purification steps. The most common methods for purification include density Percoll gradient centrifugation to remove cellular debris, red blood cells and cells of high density such as leukocytes and cell conglomerates [9, 12]. Additionally, some protocols employ a modified medium and magnetic beads covered by laminin—substrate of the extracellular matrix [13].

Some authors have adapted more accurate immune-based purification protocols for the elimination of non-trophoblast cells [10, 14–16]. Methods of cell separation on immunomagnetic beads include the negative selection of HLA class 1a positive cells, or a positive selection of CD326 positive trophoblasts [9, 17]. Since CD9 is a marker of placental mesenchymal cells, placental macrophages and blood monocytes but not trophoblasts, negative selection by anti-CD9 immunomagnetic purification can also be used [18]. Concurrently, many factors can affect the quality of the isolated cells. For example, it has been shown that the composition and characteristics of trophoblast cell populations isolated via the method of enzymatic digestion, together with negative selection alongside anti CD9-coupled magnetic beads, depends on the age of the placenta and the time of trypsinization [17]. Purification by flow cytometry can be performed to further purify trophoblast cells from side populations [11].

A large variety of methods and approaches make it difficult to choose a method for the isolation of a pure population of villous trophoblasts from human placenta that will be both efficient and reproducible. The aim of our work was to obtain a homogeneous population of human term placenta cytotrophoblasts that would be able to form a confluent monolayer and to study their characteristics in culture.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and DMEM/F12, L-glutamine, bovine serum albumin (BSA), trypsin, antibiotics PenStrep (Penicillin–Streptomycin), phosphate-buffered solution (PBS), Papanicolaou's stain OG6 and Papanicolaou's solution EA50, Percoll, trypan blue solution, red blood lyses buffer, fixation buffer and a permeabilization wash buffer were purchased from Sigma-Aldrich (USA). Foetal bovine serum (FBS, Atlanta Biologicals, USA), Hoechst-33258 fluorescent stain (Life Technologies, cat. no. 62249), DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (Thermo-fisher scientific, USA, cat.no D1306) and deoxyribonuclease I type (DNAse I, Invitrogen, USA) were used.

Mouse IgG1 anti-cytokeratin 7 (RCK105) antibodies were purchased from Thermo Scientific (USA), mouse anti-E-cadherin primary antibodies (MAB1838) from RND Systems (USA) and mouse PE- conjugated anti-CD45 [IM1833] from Beckman Coulter (USA). Rabbit anti-Vimentin [EPR3776], mouse anti-CD9 [ALB6], anti-HLA Class1 (MEM 81), FITC-conjugated anti-CD9 [MEM 61] primary antibodies, as well as rabbit FITC-conjugated anti-mouse secondary antibodies were obtained from GeneTex.co. (USA). Goat FITC-conjugated anti-rabbit secondary antibodies were purchased from Life Technologies (USA). Goat FITC-conjugated anti-mouse [RB-1930-R2], DyLight594-conjugated anti-rabbit [18249], HRP-conjugated anti-mouse [32230] and HRP-conjugated anti-rabbit [32260] antibodies were obtained from ThermoScientific (USA). All chemicals were used directly without further purification. Antibodies were diluted according to manufacturer specifications.

Ethic approval

This study was approved by the Review Board and Ethics Committee of the Federal State Budget Institution, "Research Center for Obstetrics, Gynecology and Perinatology" (Moscow, Russia). Normal human term placentas used for primary trophoblast isolation were obtained by caesarean section at 37–39 gestation weeks after informed consent was given. All procedures involved in the collection of placentas and their transportation were performed under aseptic conditions.

Methods

Isolation protocol

Human term placentas were collected from caesarean deliveries and the time from delivery to cell isolation did not exceed 60 min. After a visual inspection, a foetal part of placenta to a depth of several millimetres and containing microvillous tissue was isolated. Villous tissue, separated from blood vessels and connective tissue using a scalpel, were rinsed thoroughly in PBS and cut into small pieces.

Isolation of single trophoblast cells (*method 1*) The microvillous placental cells were isolated using a three-time treatment of obtained tissue in enzyme digestion solution containing 0.25% trypsin and 0.1 mg/ml DNAse I at 37 °C in shaking water bath (for 15–20 min at every cycle). Cell suspension was collected in a 50 ml test tube after each cycle and the remaining tissue was digested in the fresh enzyme solution. After the third cycle of digestion enzymes were inactivated by adding an equal volume of DMEM medium containing 10% FBS.

Collected cells were then filtered through a 100 µm strainer (Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 350*g* for 10 min at 4 °C. Cell pellets were resuspended in 5 ml of complete DMEM, layered on the top of a preformed Percoll gradient (65, 55, 50, 45, 35, 30 and 25%) and centrifuged at 730*g* at 4 °C for 30 min. The layer containing trophoblast cells was collected between the 45% and 35% Percoll aliquots (density 1.050–1.060 g/ml), resuspended in complete DMEM and centrifuged at 350*g* at 4 °C for 10 min. The resulting cell pellet was resuspended in PBS, cells were calculated and the suspension was re-centrifuged at 350*g* at 4 °C for 10 min.

Freshly isolated cell suspension was plated on a growth surface of Petri dishes at a concentration of no less than 5×10^5 cells/ml of nutrient medium DMEM/F12, enriched with 7% FBS, penicillin (10 U/ml)/streptomycin (10 µg/ml) and cultured at 37 °C in 5% CO_2. After 5–12 h, the non-adherent cells were removed by gentle washing. Following on, the culture medium was replaced every 2–3 days; cell phenotype and confluence were controlled under an inverted microscope (Nicon, EclipseTI-S, Japan) daily.

Isolation of trophoblasts from enzymatic-explants (*method 2*) The villous explants were received from pellets of tissue pieces, left after method 1 processing, after enzymatic digestion by washing the tissue twice in PBS with the addition of 5% FBS and centrifugation at 350*g* in 4 °C for 10 min; 2–3 ml of culture medium DMEM/F12 supplemented with 7% FBS and antibiotics were added to the resulting pellet and explants were transferred to Petri dishes and cultured under standard conditions in a CO_2 incubator (37 °C, 5% CO_2). After 7–14 h, gently (not shaking) and without taking adherent tissue pieces, the culture medium was added to a volume of 10 ml. The next day and then every 3–4 days, a partial (up to 50–75% v/v) changing of medium was performed. Control of cell growth was carried out under an inverted microscope. For isolation of trophoblasts, we used cells that formed islands of epithelial or like the "crazy pavement" morphology around explants. Under an inverted microscope in aseptic conditions, sterile glass cloning cylinders ($V = 150$ µl, C1059, Sigma-Aldrich, USA) were adjusted on these outgrowth islands, cells were removed with a 0.25% trypsin solution and transferred to a new culture flask at a concentration of $2–5 \times 10^5$ cells/ml. Cell isolation using glass cloning cylinders is shown in Fig. 1. As a control, a culture of cells isolated from the islands with mixed cell morphology and choriocarcinoma trophoblast cell line BeWo [19] were used.

Characterization of cells

Cell morphology The isolated live trophoblast cells were regularly controlled under the inverted microscope. To control cell morphology, cultures were fixed in 4% paraformaldehyde and stained with haematoxylin (Papanicolaou stain). Culture microphotographs were taken with a digital camera (Nicon, Japan).

The immunophenotype of cells and specific markers were studied by immunocytochemical and immunohystochemical staining (ICC and IHC), as well as by flow cytometry. Cells for ICC and IHC were cultured on glass chamber slides (Permanox Chamber Slides, NalgeNunc) and were fixed in 4% paraformaldehyde (20 min, 4 °C).

Immunocytochemistry staining (*ICC*) Fixed cells were incubated with mouse or rabbit primary antibodies to Cytokeratin 7 (CK7), vimentin, E-cadherin or CD9 (1 h, 20–25 °C). After washing (3 × 5 min) with PBS, cells were incubated with FITC- or DyLight594-conjugated species-specific secondary antibodies for 1 h at 4 °C in darkness. The nuclei were post-stained with fluorescent dye DAPI or Hoechst 33258 (10 min, 25 °C). Cells were analysed in visible and UV light and fluorescent images of stained cells were obtained using an Olympus Fluoview FV10 confocal microscope (Olympus, Japan).

For *immunohistochemical analysis* (*IHC*), cells in the monolayer were fixed and incubated with primary antibodies to CK7 and vimentin, and then with HRP-conjugated species-specific secondary antibodies, according to the manufacturer's instructions, and a routine colour development procedure was performed with DAB (3′3′diaminobenzidine) as chromogen and haematoxylin counterstain. The preparations were analysed in visible light under an inverted microscope (Nikon, Eclipse TI-S, Japan).

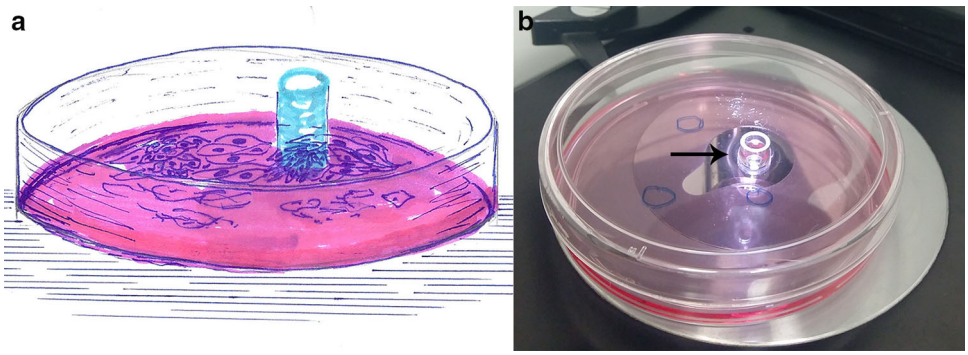

Fig. 1 Isolation of trophoblasts from outgrowth cell islands using a glass-cloning cylinder represented in a scheme (**a**) and on Petri dish (**b**). The *arrow* indicates the glass-cloning cylinder

Flow cytometry analysis The expression of surface markers (CD9, CD45 and HLA-1) was studied according to the supplemental data to flow cytometer (FC500, Beckman Coulter, USA). Following trypsinization and centrifugation (7 min, 100*g*), cells were resuspended in 400 µl of PBS with 1% FBS and 100 µl aliquots of the cell suspension were prepared. Antibodies to CD9, CD45 and HLA-1 conjugated with fluorescent label FITC (Beckman Coulter) were added to samples and incubated in darkness (15 min, 25 °C), according to the manufacturer's instructions. Following centrifugation (5 min, 400*g*), the pellets were resuspended in 1 ml of PBS containing 1% FBS and transferred to tubes optimized for the flow cytometer. The obtained data were processed using CXP software.

Results

Cell isolation

Culturing of isolated trophoblast cells (method 1)

Primary cultures of villous trophoblasts were presented by epithelial-like mononuclear cells. At 2–5 days after initial seeding, cell division with the formation of trophoblast-like cell islands was observed (Fig. 2a, b). In the following 3–5 days, cell actively differentiated by fusion to form syncytiotrophoblasts, containing between 2 and 10 nuclei (Fig. 2c, d). By the end of 7–9 days in culture, more than 50% of cells had fused into syncytiotrophoblasts. These multinuclear cells did not divide in the future and did not form a confluent monolayer.

Trophoblasts from microvillous enzymatic-explants (method 2)

Following enzymatic digestion, microvilli were presented by loose tissue consisting of cells non-separated from each other (Fig. 3). After placing explants in floating conditions in a culture medium, cells began to aggregate and formed spheroid-like structures (Fig. 3a). Immunocytochemical analysis revealed positive expression of cytotrophoblast-specific marker CK7 in the surface cells of these spheroids (Fig. 3b).

Microvillous enzymatic-explants placed on Petri dishes in a small volume of culture medium adhered to the plate surface within 6–10 h and after 2–3 days, active cell division was observed around them. The outgrowth islands consisted of cells with different morphologies: there were fibroblast-like, epithelial-like and mixed cell types, ranging from 20 to 90 µm (in the monolayer) (Fig. 4). Cells were primarily mononuclear, but a small number of polynuclear cells also appeared.

The islands of epithelial-like, oval or spindle-shaped cells—trophoblasts that formed a confluent monolayer—were the primary interest of this study. Cell islands grew and by day 10 in culture reached one, two or more centimetres in diameter, with cells retaining the characteristics of trophoblasts, becoming epithelial-like, or later changing their morphology to spindle-shaped, with one or two nuclei, and later forming the monolayer that was earlier described as the "crazy pavement" [16]. These cells were isolated using glass cloning cylinders and at the next passage, they formed a loose net-like monolayer of mononuclear round, oval or spindle-shaped cells up to 40–80 µm in length. After 5–7 days in culture, cells formed the dense monolayer resembling the "crazy pavement" (Fig. 5). Most cells in culture were elongated and varied in size, from 30 to 70 µm; in suspension, however, the diameter of rounded cells was roughly 10–20 µm. The appearance of polynucleated cells, the number of which in obtained cultures varied from 3–5 to 10–15%, were observed.

Immunophenotypic characterization of isolated trophoblast cells

Immunophenotype of the well-known choriocarcinoma trophoblast cell line BeWo (used as a positive control) and of the primary trophoblast culture obtained by methods 1 and 2 was similar and showed a positive expression of trophoblast-specific intracellular marker CK7 in all cells (100%) (Supplemental Fig. 1a, c, e), as well as the absence

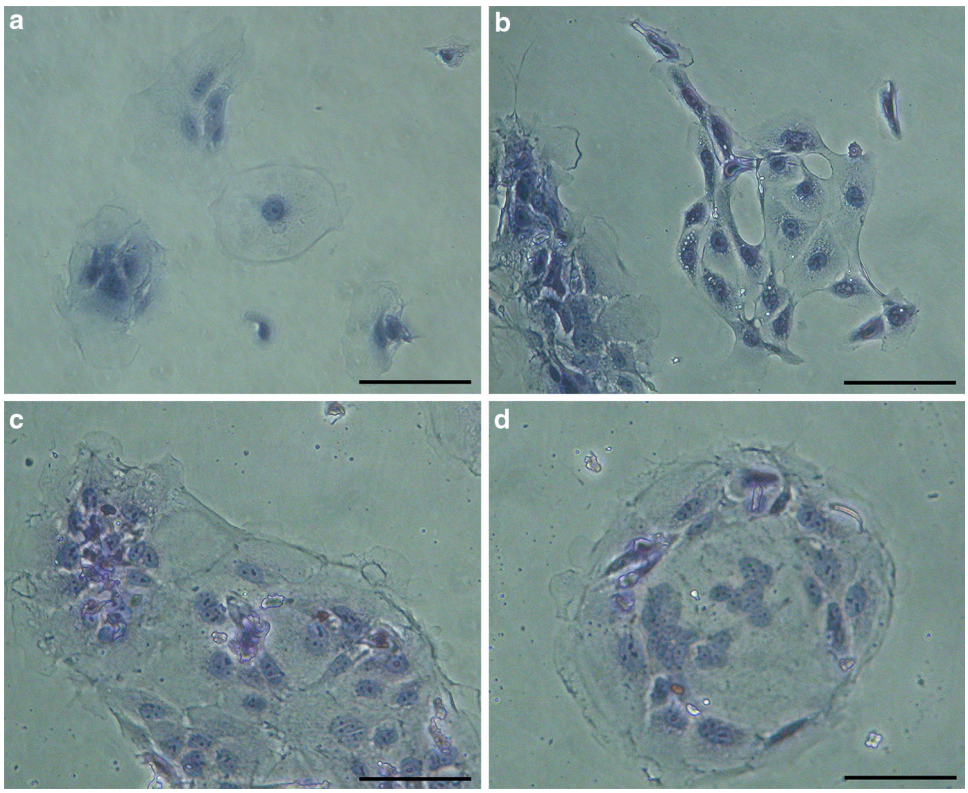

Fig. 2 Cyto- and syncytiotrophoblasts in primary culture (method 1). Villous trophoblast cells after different periods of cultivation in vitro: 2–3 days (**a**), 5 days (**b**); fusion of cytotrophoblasts and formation of polynucleated syncytiotrophoblasts after 7–9 days in culture (**c**, **d**). Light microscopy, haematoxylin stain, *scale bar* **a**, **b** 50 μm, **c**, **d** 100 μm

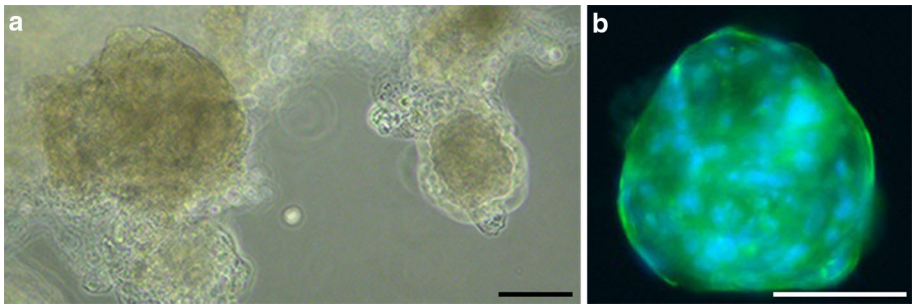

Fig. 3 Placental villous tissue explants after enzyme digestion. Microvillous explants after enzyme digestion were placed in a culture medium. Explants that were floating in the medium after 24 h formed spheroids (**a**) positively stained against CK7 (**b**). **a** Light microscopy, ×10; **b** immunocytochemical staining (FITC-conjugated CK7), nuclei stained with DAPI, laser scanning confocal microscopy, *scale bar* 100 μm

of mesenchymal marker vimentin expression in 99% or more of cells (Supplemental Fig. 1b, d, f). Cells in the primary culture obtained by method 2 remained CK7-positive and vimentin-negative following the next passage in vitro. In contrast, culture obtained from islands containing different cell types was represented by the mixture of cells positively or negatively stained against CK7 and vimentin (Supplemental Fig. 1g, h). Cytotrophoblast cells isolated by the new method (>99.9%) demonstrated positive expression of E-cadherin and CD9 negative staining (Fig. 6). E-cadherin was located in intercellular connections.

Cell control by flow cytometry confirmed that this trophoblast cell culture was contaminated with other placental cell types. The culture of mixed villous cells contained 94.3% cells positive to FITC-conjugated CD9 and 29.7% cells positive to HLA class 1 antibodies; the presence of haematopoietic cells was negligible—the number of $CD45^{+}$ cells did not exceed 2% (Fig. 7a–c).

The number of cells expressing CD9, CD45 and HLA class 1 in the culture obtained by the new method (method 2) was comparable to that in the negative control (Fig. 7d–f) and represented 0.1–0.8%. The presence of more than 99% of CD9, CD45 and HLA class 1 negative cells

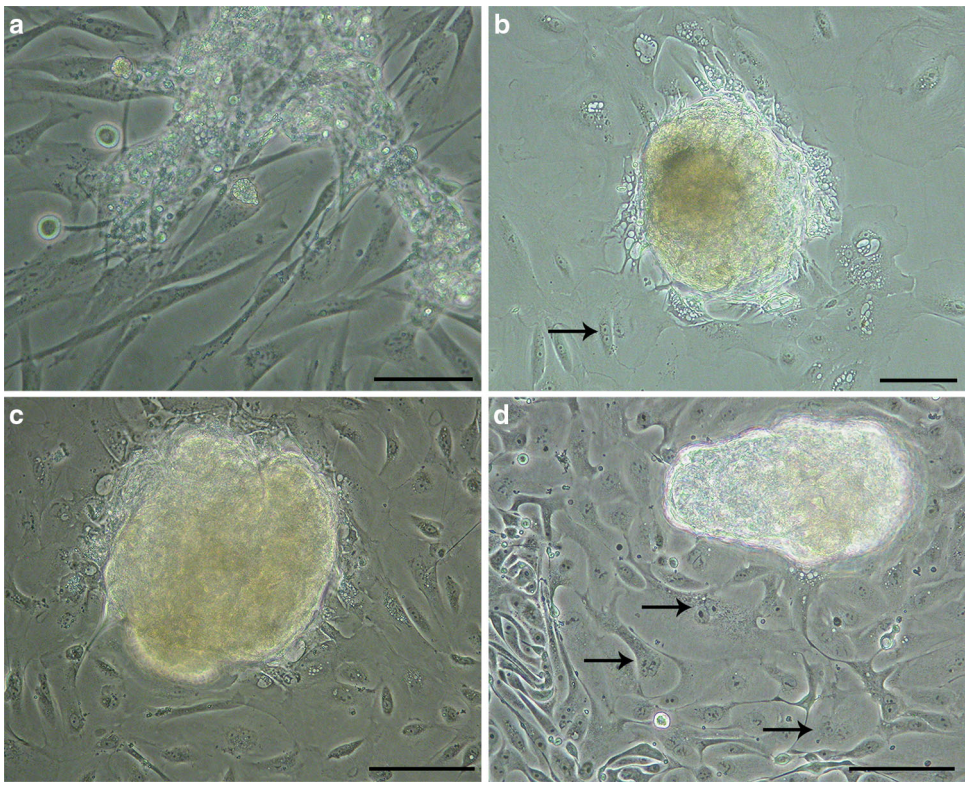

Fig. 4 Outgrowth of cells from human villous enzymatic-explants after 10 days in culture (method 2, 1 passage). **a** migration of mesenchymal-like cells; **b**, **c** outgrowth of trophoblast-like cells; **d** outgrowth of cytotrophoblasts forming a "crazy pavement" monolayer. *Arrows* indicate polynuclear trophoblasts. Light microscopy, *scale bar* 50 μm

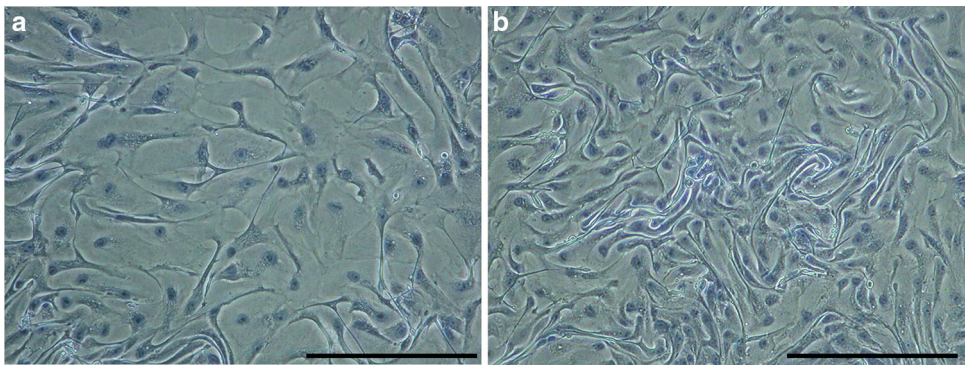

Fig. 5 Morphology of trophoblasts isolated from enzymatic-explant culture at the second passage in vitro. **a** 3 days in culture (×40); **b** 5–7 days in culture (×10). Light microscopy, *scale bar* 100 μm

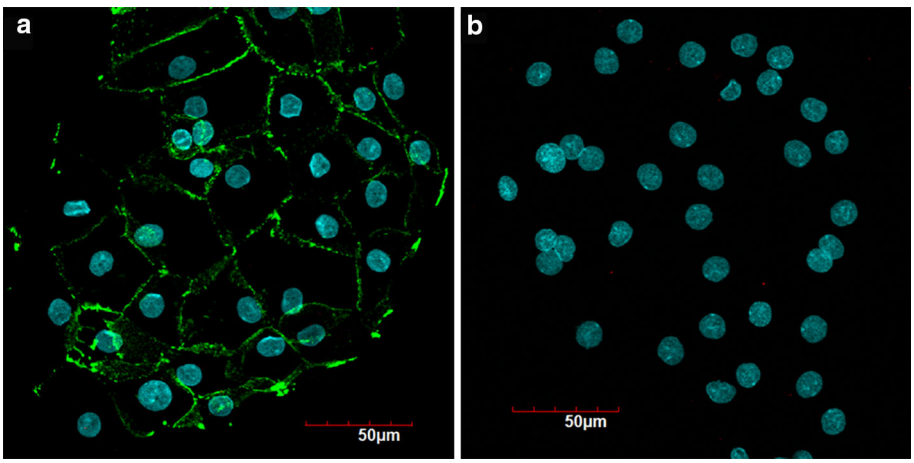

Fig. 6 Positive E-cadherin (**a**) and negative CD9 (**b**) expression in trophoblast cells isolated by the new method. Immonocytochemical staining (FITC-conjugated antibodies), nuclei stained with DAPI, laser scanning confocal microscopy

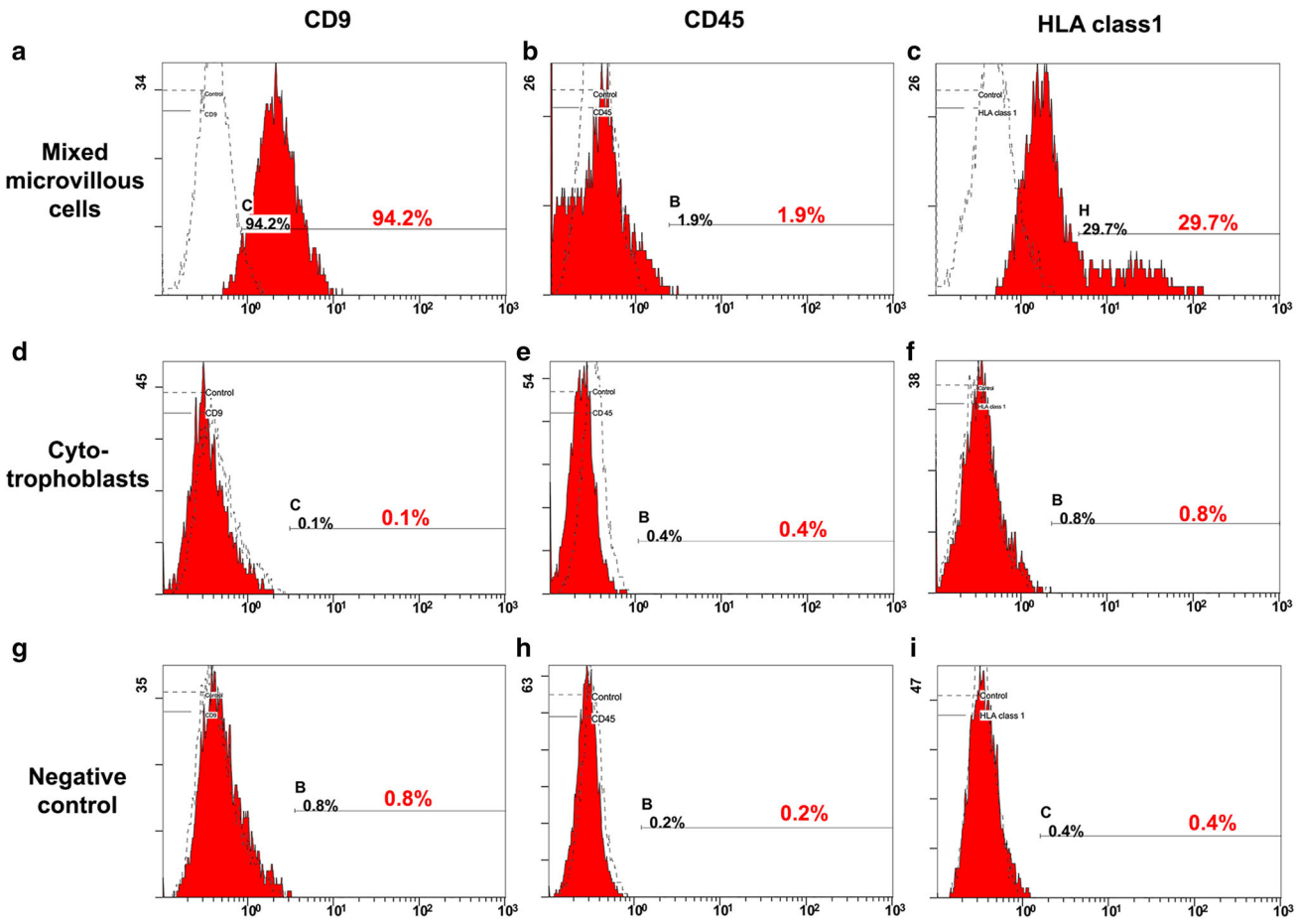

Fig. 7 Flow cytometry analysis of surface markers CD9, CD45 and HLA class 1 expression in isolated cytotrophoblast cells. The expression of CD9, CD45 and HLA class 1 was studied in a mixed microvillous cell culture (**a–c**), in a cytotrophoblast culture isolated by the new method (**d–f**) and in a negative control culture (**g–i**). The *dashed line* represents FITC-conjugated IgG isotype control

confirmed the high purity rate of the obtained cytotrophoblast cell population (Fig. 7g–i).

Discussion

The results of the current study indicate that trophoblast primary cell cultures isolated using the traditional method exhibited epithelial-like cells that rapidly lost their capacity to proliferate and that spontaneously differentiated into polynuclear syncytiotrophoblasts, which is in accordance with previously conducted studies [20]. Cervar-Zivkovic and Stern showed that numerous genes associated with syncytiotrophoblast differentiation were upregulated in cytotrophoblast cells isolated from villous term placenta aggregates within four hours after placing them in a culture medium containing foetal calf serum (FCS) [10]. After 24 h in culture, >95% of cells were mononuclear and within 72 h, single cells fused and formed syncytium [10]. These cells may, therefore, be of practical use for studying the release of syncytial hormones and factors in vitro [17], or for studying signalling mechanisms of trophoblast differentiation [5]. However, cells isolated using this method cannot be maintained in culture for a long period of time, do not form a confluent monolayer and cannot be passaged.

The proposed method for obtaining cytotrophoblasts from outgrowth islands of placental microvillous tissue enzymatic-explants isolated with glass cloning cylinders allowed us to obtain a pure population of cytotrophoblasts that formed characteristic resembling the "crazy pavement" morphology confluent monolayer. Cells obtained via this method maintained morphological and cultural characteristics for at least two passages in vitro.

The morphology of cells and the monolayer were consistent with characteristics of cytotrophoblast cultures described previously [17]. The described changes in cell morphology from epithelial and oval to spindle-shaped cells support the observations of Bischof and Irminger-Finger, who showed that cytotrophoblasts are "chameleon" cells with variability and diversity in terms of morphology. It is assumed that cytotrophoblasts are not only able to fuse to form syncytia, but can also behave like

immotile polarized epithelial cells, stromal fibroblasts or endothelial cells, or undergo a mesenchymal-like transformation that converts them into highly invasive cells [21].

It is known that Cytokeratin 7 and E-cadherin, which are specifically expressed by the cytotrophoblast cells, are the best markers for the identification of isolated trophoblasts. Extravillous trophoblasts and other types of placental cells are vimentin positive cells; for this reason, these markers were chosen to control the phenotype of villous and extravillous trophoblasts as recommended by [22, 23], to assess the purity of trophoblast cell cultures isolated by the newly created method 2. In accordance with this recommendation, the presence in our culture of 100% CK7 and E-cadherin-positive, and more than 99% vimentin-negative cells, as well as the number of CD9, CD45 and HLA class 1 positive cells at the level of negative control, indicates the absence of mesenchymal and haematopoietic cells. Specific distribution of E-cadherin expression at the site of intercellular connections confirmed the epithelial origin of cells. These results confirm the purity of the villous cytotrophoblast cell culture obtained via the new method.

As far as amnion cells, fibroblasts, macrophages and endothelial cells, stain positively against vimentin, the absence of vimentin expression in isolated cells again confirmed that the isolated population was pure and not appreciably contaminated by other cell types. The presence of polynucleated cells in the culture indicated that cultured cytotrophoblasts retained the ability to differentiate into syncytiotrophoblasts.

The explant culture method has been known for some time. Placental explant 3D cultures in vitro are used for studying tissue functions, including cellular uptake, the production and release of secretory components, cell interactions, proliferation, growth and differentiation, gene delivery, pharmacology, toxicology and disease processes [24]. For obtaining a cell monolayer from tissue explants, substrates such as collagen I or an extracellular matrix (ECM), e.g., Matrigel, are generally used [25, 26]. The experience of cytotrophoblast cell isolation from explants established in tissue culture on plastic showed that these cultures are frequently contaminated with fibroblasts [27]. The control of cell morphology under a microscope allowed us to isolate a term placenta villous or extravillous trophoblast culture with a high rate of purity and without contaminating cell types.

It is known that low-differentiated progenitor cells migrate from the tissue onto the surface of the dish. Explant culture can also refer to the culturing of the tissue pieces themselves, where cells are left in their surrounding extracellular matrix. This type of culture is used because the structural integrity of multicellular organisms depends upon the establishment and maintenance of stable cellular connections [28]. Gerami-Naini et al. established a model for obtaining trophoblasts from aggregates of human embryonic stem cells (hESC), that is, embryoid bodies (EBs) [29]. Adherent cultures of these EBs led to the outgrowth of cells with an epithelial morphology that maintained hCG, progesterone and steroid hormone secretion. This model also confirmed the important role of the surrounding extracellular matrix [5, 29, 30].

It can be assumed that the observed outgrowth of cytotrophoblasts from villous enzymatic-explants and their low differentiation into polynucleated syncytiotrophoblasts are due to the formation of the extracellular matrix and the maintaining of stable intercellular connections, which provides structural and biochemical support to the surrounding cells, cell adhesion and cell-to-cell communication and differentiation, as described earlier [28–30]. An important aspect in maintaining or further differentiation, as well as in epigenetic regulation, is the presence of signalling and transcriptional factors, which are particularly important in the context of transition from 3D to 2D culture conditions [5]. It is possible that culturing single cells in the absence of intercellular junctions and a formed extracellular matrix will lead to rapid cell differentiation, whereas partial cell detachment and maintenance of intercellular contacts and signalling pathways, characteristic of tissue type. Cytotrophoblasts growing in a monolayer retain their phenotype and plasticity. In our experiments, in a two-dimensional adherent culture of villous tissue samples, we isolated the population of cytotrophoblast cells, which were CK7, E-cadherin positive and vimentin negative stained, and retained the ability to differentiate into syncytiotrophoblasts.

Conclusion

In this paper, we described a new method for the isolation of cytotrophoblasts from normal term placenta. The cultivation of trypsinized micro-pieces of placental villous tissue explants, followed by the isolation of cells from outgrowth islets, allowed for receiving a subpopulation of cytotrophoblasts free of contamination by other types of cells. Compared to other methods, our protocol is mild, simple and effective, does not include using antibodies for separation, and yields a highly pure and viable culture of villous cytotrophoblasts. The purity and cytotrophoblast nature of all isolated cells was demonstrated by positive expression of CK7, E-cadherin and negative expression of CD9, CD45, HLA class 1 and vimentin. Cells isolated by this method preserved the characteristic immunophenotype and formed a confluent monolayer following the second passage in vitro.

Cytotrophoblast cell cultures obtained via this method can be used as a model or tool for fundamental research

that focus on the influence of different culture conditions and factors on trophoblasts and other placenta cell types' growth and differentiation, as well as for the study of a variety of other aspects of placental function and their role in metabolism and drug transportation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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