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Isolation of villous cytotrophoblasts from second trimester human placentas

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Abstract

Human placental functional studies are often performed using immortalized trophoblast cells and cell lines established from human choriocarcinomas, which, while practical, may not truly reflect trophoblast function *in vivo*. Primary trophoblast cultures derived from human placentas following pregnancy termination or delivery are more clinically relevant, but trophoblast isolation protocols are only available for 1st trimester and term placental tissues. Here we report a method for isolation and purification of primary villous cytotrophoblasts from 2nd trimester human placentas, that yields >99% trophoblast purity as shown by immunofluorescence.

Keywords

trophoblasts; placenta; second trimester; primary cell isolation

Introduction:

Impaired placental development and function have been implicated in a continuum of pregnancy complications [1] and fetal programming of chronic disease across the life course [2]. The essential role of the placenta in pregnancy is unquestionable, yet despite many years of research we still do not have a complete understanding of its development and function in health and disease and in response to environmental exposures. This is in part due to the lack

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of appropriate functional models of trophoblasts, specialized cells of the placenta that play a key role in the cross talk between the mother and fetus, and whose proper function is critical for a healthy pregnancy[3]. Villous cytotrophoblast stem cells, the undifferentiated trophoblasts, proliferate and give rise to villous (CTB) and extravillous (EVT) cytotrophoblasts. The villous cytotrophoblasts further fuse to form an outer syncytiotrophoblast (STB) layer. Until recently, cellular models of trophoblast function were based on human choriocarcinoma or immortalised cells[4], which, while practical, do not functionally truly reflect a healthy trophoblast population. There are a number of different methods for isolating primary trophoblasts from 1st trimester and term placentas [5–8] in routine use, but there are no published methods for isolating cytotrophoblasts from 2nd trimester placentas. Given that the placenta undergoes dynamic structural and functional changes across gestation, and that maternal blood flow to the placenta is initiated only after 10 weeks' gestation, 2nd trimester trophoblast models would be highly informative. However, neither 1st trimester nor term published trophoblast isolation protocols were found to be suitable for 2nd trimester tissues in our hands. This is because, like 1st trimester tissues, 2nd trimester placentas are highly susceptible to over-digestion and also contain abundant fetal vasculature like term tissues, requiring further purification. We have optimised a trophoblast isolation protocol for 2nd trimester placentas that results in high purity and viability.

Methods:

Second trimester human placentas (13–24 weeks' gestation) were obtained with informed consent from women undergoing elective terminations at the Pregnancy Advisory Centre, Queen Elizabeth Hospital, Woodville, Adelaide. Ethics approval was obtained from the hospital Human Research Ethics Committee, TQEH/LMH/MH (Q20160305). Placentas were obtained within minutes of termination, non-villous tissue removed and remaining villous tissue washed with 0.9% NaCl, then transported to the laboratory in 1x PBS on ice. Between 8–25 grams of placental villous tissue (size depends on gestation) was digested in 10 mL digest buffer (1x PBS containing 0.25% trypsin and 200µg/ml DNase I) per gram of tissue. Villi were incubated at 37°C for 10 min then washed 10 times with 20 mL 1x PBS to remove STBs.

Following the initial digest to remove STBs and EVTs, remaining villous tissue underwent a second digest, this time at 4°C with gentle rocking for 7 min then stationary incubation overnight at 4°C. The next day, the supernatant was filtered through a 70 µm cell strainer into 50 mL tubes containing 10% FBS (final concentration) to inactivate trypsin. Remaining tissue was washed ten times with 1x PBS and all resuspended cells from each wash filtered in the same way. Collected cells were centrifuged at 450g for 8 min, then all cell pellets were combined into a single 50 mL tube, re-centrifuged and resuspended in 6 mL 1x Ca/Mg free HBSS. 4 mL of cell suspension was loaded onto two Percoll gradients (70–5%; pre-prepared in 50 mL tubes using 1x HBSS). Samples were centrifuged at 1200g for 20 min without brake applied. Trophoblast cells located between 15–27 mL layers of the 50 mL tubes were collected and washed by gentle inversion with 4x volume of DMEM containing 1% antibiotic/antimycotic and 10% FBS. Cells were centrifuged at 1000g for 10 min and the resulting pellet resuspended in DMEM.

Negative selection was performed on resuspended cells to remove mesenchymal cells and leukocytes. Cells were incubated with CD9 and CD45 antibodies (10 µg each antibody per 10⁷ cells) for 10 min at 4°C with gentle rotation. Cells bound to antibodies were washed in 4x volume of 0.1% BSA/PBS then centrifuged for 5 min at 1000g. Cell pellets were resuspended in 0.1% BSA/PBS at 10⁷ cells/mL, with 50 µL pan anti-mouse Dynabeads. Non-trophoblast cells were separated using a magnet, leaving a pure trophoblast population in the eluate. A subset of cells was immobilised to slides by cytopsin (Shandon, CytoSpin 4) centrifugation to assess purity by immunofluorescence. The following antibodies were used to identify different cell populations: VIM and CD9 (fibroblasts, leukocytes, endothelial cells [9]), CD45 (leukocytes), HLA-G (EVTs), KRT7 (all trophoblasts), PEG10 (CTBs) and PSG1 (STBs). All materials and antibodies used are listed in Table 1.

Results and Discussion:

Here we present an optimised method for isolating villous cytotrophoblasts from 2nd trimester human placentas. This isolation protocol yields a highly pure and viable trophoblast population (Figure 1), comprising mostly CTBs (identified by PEG10 positivity [10, 11]). VIM+ cells made up less than 0.2% of the final cell population while less than 0.1% of the cells were positive for CD45. More than 99% of the cells were KRT7 positive, identifying an almost pure trophoblast population. Because these cells could be either CTBs, EVT's or STBs, we assessed them for trophoblast specific markers. We found no evidence of STB presence (as indicated by the lack of staining for PSG1); less than 1% of the cells were positive for HLA-G, indicating a small presence of EVT's and more than 99% of the trophoblast cells were positive for PEG10, indicating a highly pure CTB population.

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- 1st trimester CTB isolation protocols are unsuitable for 2nd trimester tissue
- Term placental CTB isolation protocols are also unsuitable for 2nd trimester tissue
- Our protocol yields a highly pure CTB population from 2nd trimester placentas

Figure 1:

Characterisation of cell populations isolated from 2nd trimester human villous placental tissues. (A) 2nd trimester placental villous tissue as an example of antibody target specificity (PSG1 (green) is STB specific, PEG10 (red) is CTB specific; nuclei are labelled with DAPI (blue)). (B, C) KRT7 positivity indicates >99% trophoblast purity; (D, E) PEG10 positive cells indicate the presence of a highly pure CTB population; (F, G) HLA-G positivity shows <1% extravillous trophoblasts; (H, I) VIM and (J, K) CD45 positive cells indicate a very small presence of co-isolated fibroblasts and leukocytes. (B, D, F, H, J) represent single

channel images of individual protein markers, (C, E, G, I, K) images merged with DAPI, identifying individual nuclei. 40x magnification (A), 20x magnification (B-K); scale bar represents 20 μm (A), 50 μm (B-K).

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Table 1.

Reagents and antibodies used for isolation of cytotrophoblasts from 2nd trimester human placentas.

Materials	Company	Catalogue #	Notes
1x PBS	Life Technologies	10010031	
0.25% Trypsin	Merck	59427C	
DNase I	Merck	04536282001	
Percoll	Merck	P4937	
1x HBSS	Life Technologies	14170161	
DMEM	Life Technologies	10566016	
70um cell strainer	Merck	CLS431751	
50mL tube	McFarlane Medical	31301BD	
Antibiotic/Antimicotic	Merck	15240062	
Fetal bovine serum (FBS)	Merck	F9423	
BSA	Merck	A9418	
Dynabeads™ Pan Mouse IgG	Life Technologies	11042	
CD45	Invitrogen	14-0459-82	Clone # HI3 Dilution 1:100
KRT7	Dako	M7018	Clone # OV-TL 12/30 Dilution 1:200
HLA-G	Santa Cruz	Sc-21799	Clone # 4H84 Dilution 1:100
PEG10	Abcam	AB215035	Clone # EPR20051 Dilution 1:100
PSG1	R&D Systems	MAB6799	Clone # 684701 Dilution 1:100
VIM	Dako	M7020	Clone # 3B4 Dilution 1:50
CD9	R&D Systems	MAB1880	Clone # 209306
Alexa Fluor 488 Anti-Mouse	Life Technologies	A-11001	Dilution 1:500
Alexa Fluor 555 Anti-Rabbit	Life Technologies	A32732	Dilution 1:500