

# Endoglandular trophoblast, an alternative route of trophoblast invasion? Analysis with novel confrontation co-culture models

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**BACKGROUND:** Routes of trophoblast invasion seem to be clear, whereas specific invasive pathways need further elucidation. Extravillous trophoblasts (EVTs) transform spiral arteries to guarantee appropriate blood flow to the placenta in the second trimester. Embryo nutrition during the first trimester is thought to be histiotrophic, whereas proof that EVT also invade uterine glands is lacking. We developed novel three-dimensional confrontation co-culture models to elucidate invasion of EVT into uterine glands.

**METHODS:** First trimester decidua parietalis and placental villous explants were directly confronted and co-cultured for 72 h, or confronted indirectly after 72 h pre-culture for re-epithelialization of decidua pieces. Cryosections were stained by immunohistochemistry or immunofluorescent/immunohistochemical double labelling and compared with first trimester placentation sites *in situ*.

**RESULTS:** EVT deeply invaded decidual tissues in direct confrontation assays and were found between the decidual epithelial cells and epithelial basement membrane. EVT were also detected in the decidual stroma in direct proximity to glands, sometimes even replacing glandular epithelial cells. Similar observations were made in sections from the first trimester decidua/placental bed. In the invaded parts of sections of decidua basalis, 55% ± 7% (mean ± SEM; n = 10, range 6–11 weeks) of glandular cross sections were associated with or infiltrated by EVT.

**CONCLUSIONS:** Using novel confrontation co-culture assays, a potential new route of EVT invasion was detected. EVT appear to break through the basement membrane of uterine glands to open their lumen towards the intervillous space. These data support the hypothesis of histiotrophic nutrition of the embryo prior to onset of maternal blood flow within the placenta.

**Key words:** confrontation / co-culture / invasion / trophoblast / uterine glands

## Introduction

At ~2 weeks after fertilization extravillous trophoblasts (EVTs) start to infiltrate maternal tissues by invading the decidual stroma. On their way through the uterine interstitium, they pass capillaries and glands, finally reaching the myometrium and/or spiral arteries. So far, trophoblast invasion has been described to serve attachment of the placenta to the uterus as well as transformation of spiral arteries (Kaufmann *et al.*, 2003). Trophoblast infiltration of the spiral arteries leads to a blockage of these vessels until the end of the first trimester, and only with the beginning of the second trimester, the maternal blood flow through the intervillous space of the placenta is established, allowing the haemotrophic nutrition of the embryo (Hustin and Schaaps, 1987; Jaffe *et al.*, 1997; Jauniaux *et al.*, 2000; Kliman,

2000). This poses the question of how the embryo receives nutritional input during the first trimester of pregnancy.

The endometrium as well as the pregnant decidua contains a huge number of uterine glands that open towards the uterine cavity. Their secretion products play an essential role in feeding and maintaining the very early embryo prior to implantation (Burton *et al.*, 2002). It is known from other eutherian species that such secretion products are a mixture of proteins, lipids and carbohydrates (Amoroso, 1952). Recently, their role has been extended from purely nutritional to also being immunosuppressive or controlling the whole process of implantation since they contain leukaemia-inhibitory factor and MUC-1 (Seppala *et al.*, 1998; Carson *et al.*, 2000; Brayman *et al.*, 2004).

Until recently, it was assumed that glandular secretion products do not play a role in nutrition of the human embryo following

implantation. However, a recent study has demonstrated their presence in the intervillous space of the first trimester placenta. Burton *et al.* (2007) demonstrated that the clear fluid found in the intervillous space of a first trimester placenta contains secretory components of the uterine glands. It is, however, unclear as to how these glandular products reach the intervillous space of the placenta. It may be speculated that the invasive EVT populations that target the uterine spiral arteries and initiate maternal blood flow into the intervillous space play a similar role in the opening of uterine glands.

Co-culture model systems using decidual tissues as matrices for trophoblast invasion have been used successfully for answering various questions concerning invasion of trophoblast cells, such as maternal blood vessel remodelling (Dunk *et al.*, 2003), or effects of cytokines and growth factors (Helige *et al.*, 2001).

Here, we present novel confrontation co-culture model systems to analyse trophoblast invasion into the decidual interstitium and towards uterine glands. The data derived from the model systems may provide first evidence for the infiltration of the lining of the glandular epithelium by EVT, thus opening the way for secretion products to reach the intervillous space.

## Materials and Methods

### Tissue collection

First trimester placentas were obtained from elective terminations of pregnancies [gestational age (GA) 5–11 weeks,  $n = 36$ ]. Invaded decidual basalis was obtained from 10 placentas with GA of 6, 7, 8, 9 and 11 weeks. For the *in vitro* experiments, only samples from placentas with a GA of 6–9 weeks were used ( $n = 31$ ). Informed consent was obtained from each patient with approval of the local ethical committee. Tissues were rinsed in Hank's buffered salt solution, supplemented with 1% penicillin/streptomycin and 1% amphotericin B (PAA Laboratories, Austria), placed in culture media (Dulbecco's modified Eagle's medium high glucose with sodium pyruvate, without L-glutamine, supplemented with 10% fetal calf serum, 2% L-glutamine, 1% penicillin/streptomycin, 1% amphotericin B (PAA Laboratories), 20 ng/ml progesterone and 3 ng/ml 17- $\beta$ -estradiol (Schering, Germany) and prepared for culture under a dissecting microscope. Only chorionic villi and decidual parietalis with intact epithelium were selected from the tissue sample and dissected in culture media. Absence of invasive trophoblast cells from the selected decidual parietalis was routinely checked by HLA-G immunohistochemistry. From every placenta, various tissue samples were collected for fixation in 4% paraformaldehyde and subsequent paraffin embedding. Additional tissue samples were embedded in tissue freezing medium (TissueTek<sup>®</sup>; Sakura Finetek Inc., Torrance, USA) and stored at  $-70^{\circ}\text{C}$ .

### Confrontation co-culture

Two similar confrontation co-culture assays were developed. For both confrontation assays (direct and indirect confrontation), the layer with the luminal epithelium (stratum compactum) was removed with a scalpel from the decidual stroma (stratum spongiosum) to obtain uniform pieces of tissue with equal orientation. The stroma was then dissected in uniform, round-shaped pieces of  $\sim 1$  mm diameter, and villi were cut into pieces of corresponding size (15–20 mg moist mass). For direct confrontation (without decidual epithelium), one piece of decidual was co-cultured with one villous explant in 500  $\mu\text{l}$  culture media in a 2-ml reaction tube (Eppendorf, Germany). Before culture, the reaction tubes were perforated for gas exchange. For indirect confrontation (with decidual

epithelium), decidual pieces were pre-cultured as described previously (Helige *et al.*, 2008). Briefly, decidual pieces were transferred into 25 ml spinner flasks and stirred with  $\sim 130$  rpm with a magnetic stirrer system (Telesystem 06.40 and Telemodul 40C, H + P Labortechnik AG, Oberschleißheim, Germany) for 72 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Culture media was changed once after 24 h. Villi were pre-cultured in parallel in 20 ml culture media in 10 ml petri dishes (Greiner Bio-one, Kremsmuenster, Austria) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  and 2.5%  $\text{O}_2$  in an XVIVO incubation system (Model G300C, BioSpherix Ltd, Lacona, USA). After 72 h pre-culture, the decidual pieces rounded and re-epithelialized and were confronted with the villous explants in the same way as the direct confrontations.

Villous tissue was normally confronted with decidual tissue from the same pregnancy for 72 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Optionally, villi from one placenta were pre-cultured at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  and 2.5%  $\text{O}_2$  for 72 h and then confronted with fresh decidual from another placenta. Control cultures with decidual and villous explants cultured alone were set up in parallel with each confrontation experiment.

After confrontation culture the adhered tissues were routinely embedded in tissue freezing medium (TissueTek) and processed for immunohistochemistry/immunofluorescence. To obtain a better morphology, tissues from an indirect confrontation were fixed in 4% paraformaldehyde (LaboNord, Vienna, Austria), embedded in paraffin and processed for immunohistochemistry.

### Immunohistochemistry/immunofluorescence

Serial 5  $\mu\text{m}$  sections were cut and placed in duplicates on Superfrost Plus slides (Menzel, Braunschweig, Germany). Cryosections were air dried overnight and stored at  $-20^{\circ}\text{C}$ . Every 10th slide was routinely assessed using anti-HLA-G antibodies. For immunohistochemistry and immunofluorescence, slides were thawed, air dried, fixed in acetone, air dried again for 5 min and rinsed in phosphate-buffered saline (PBS). Paraffin embedded sections were deparaffinized in xylene and rehydrated through a series of graded alcohol. Heat-induced antigen retrieval was performed in antigen retrieval solution at pH 9 (Eubio, Vienna, Austria) in a pressure cooker for 7 min at  $120^{\circ}\text{C}$  before immunohistochemistry.

Immunohistochemistry was performed using the Ultravision LP detection system (Thermo Scientific, Fremont, USA) according to the manufacturer's instructions. For cryosections Ultra V Block was supplemented with 10% human AB-serum. Primary antibodies were diluted in antibody diluent (Dako, Carpinteria, USA). Table 1 lists details of all antibodies used and their respective dilutions. Sections were counterstained with Mayer's hemalaun and mounted with Kaiser's glycerol gelatin (Merck, Vienna, Austria). Negative controls were incubated with the appropriate immunoglobulin (Ig)G fractions as isotype controls (Table 1).

Immunohistochemical double labeling was performed using the MultiVision Polymer Detection system (MultiVision anti-rabbit/AP + anti-mouse/HRP polymers; Thermo Scientific) according to the manufacturer's instructions.

For immunofluorescent, double labelling the slides were incubated with an Ultra V-Block (Thermo Scientific) containing 10% human AB-serum for 8 min at room temperature. Slides were rinsed in PBS three times before applying both primary antibodies for 30 min at room temperature. Primary antibodies were diluted in antibody diluent (Dako). Slides were rinsed again in PBS three times and were then incubated for 30 min at room temperature with 20  $\mu\text{g}/\text{ml}$  fluorescent labelled secondary antibodies (Alexa Fluor<sup>®</sup>555 goat anti-mouse IgG and Alexa Fluor<sup>®</sup>488 goat anti-rabbit IgG; both Invitrogen, Lofer, Austria). In an alternative protocol for immunofluorescent double staining, slides were incubated with a single unlabeled primary antibody for 30 min at room temperature, followed by incubation with 20  $\mu\text{g}/\text{ml}$  Alexa Fluor<sup>®</sup>555 goat anti-mouse IgG, followed by incubation with a directly fluorescein isothiocyanate conjugated anti-cytokeratin antibody. Slides were

**Table 1** Primary antibodies used in immunohistochemistry/immunofluorescence.

Antibody clone (Cat No)	Company	IHC cryo	Dilution IF cryo	IHC ffpe	Host/Isotype
Cytokeratin 7 (OV-TL 12/30)	Thermo Scientific (Fremont, USA)	1:10 000	—	1:3000	Mouse IgG mc
Cytokeratin 7 (APO6204PU-N)	Acris (Herford, Germany)	—	1:100	1:200	Rabbit IgG pc
HLA-G (MEM-G/9)	Exbio (Prague, Czech Republic)	1:2000	1:500	—	Mouse IgG mc
HLA-G (4H84)	Exbio (Prague, Czech Republic)	—	1:5000	1:10 000	Mouse IgG mc
Human Ki67 (MIB-1)	Dako (Carpinteria, USA)	1:100	—	—	Mouse IgG mc
FITC-conjugated cytokeratin (MNFI 16)	Dako (Carpinteria, USA)	—	1:5	—	Mouse IgG mc
Human Nidogen (Entactin) (BCO-A4002A-1)	Eubio (Vienna, Austria)	—	1:150	—	Rabbit IgG pc
Mouse IgG1 (DAK-GO1)	Dako (Carpinteria, USA)	1:100	1:100	1:100	Mouse IgG mc
Rabbit immunoglobulin fraction (X 0903)	Dako (Carpinteria, USA)	—	1:300	—	Rabbit

mc, monoclonal; pc, polyclonal; IHC, immunohistochemistry; IF, immunofluorescence; ffpe, formalin fixed paraffin embedded; FITC, fluorescein isothiocyanate; Ig, immunoglobulin.

counterstained with 4,6-diamidino-2-phenylindolehydrochloride (DAPI; diluted 1:2000 in PBS; Invitrogen) for 10 min at room temperature. Slides were rinsed in deionized water and mounted with ProLong® Gold antifade reagent (Invitrogen). Sections were assessed with an AxioPhot microscope and photographs were taken using an AxioCam HRc digital camera (Zeiss, Oberkochen, Germany). Invasion of EVT into glands was quantified in immuno double labelled sections of invaded decidua basalis (10 cases, 6–11 weeks). Glandular cross sections associated with or invaded by EVT were counted and compared with the total number of glandular cross sections in the invaded area of the tissue section.

## Results

### Confrontation model systems

We developed confrontation co-culture model systems for the investigation of EVT invasion into decidual tissues. The main difference between the two confrontation model systems is the presence/absence of a decidual epithelium. For direct confrontation, first trimester villous explants were placed on the top of a piece of decidua parietalis (without epithelium) and cultured for 72 h. Alternatively, decidua pieces were pre-cultured under constant agitation for 72 h for re-epithelialization and then confronted with villous explants from the same pregnancy (indirect confrontation) (Fig. 1). In both model systems, confrontation of villous and decidual tissues resulted in adhesion of tissues (Fig. 2A). The anti-HLA-G antibody MEM-G9 was used to specifically visualize EVT. Strong HLA-G immunostaining confirmed the attachment of villi to decidual tissues and the subsequent formation of trophoblastic cell columns in direct and indirect confrontations (Fig. 2B and E, respectively).

During direct confrontation (no epithelium) EVT showed strong invasion into the decidual stroma (Fig. 2B). A time course showed that first attachment with invasion of HLA-G positive trophoblast cells takes place after 8 h of co-culture (data not shown). Morphological assessment showed that the viability of the tissues is maintained throughout the culture period. There were no visible signs of necrosis even after 140 h of culture. Immunohistochemical staining with the proliferative marker Ki67 revealed the presence of proliferative cells after 72 h of culture (Fig. 2C, serial section to 2B). Isotype negative control antibodies did not reveal any staining (Fig. 2D).

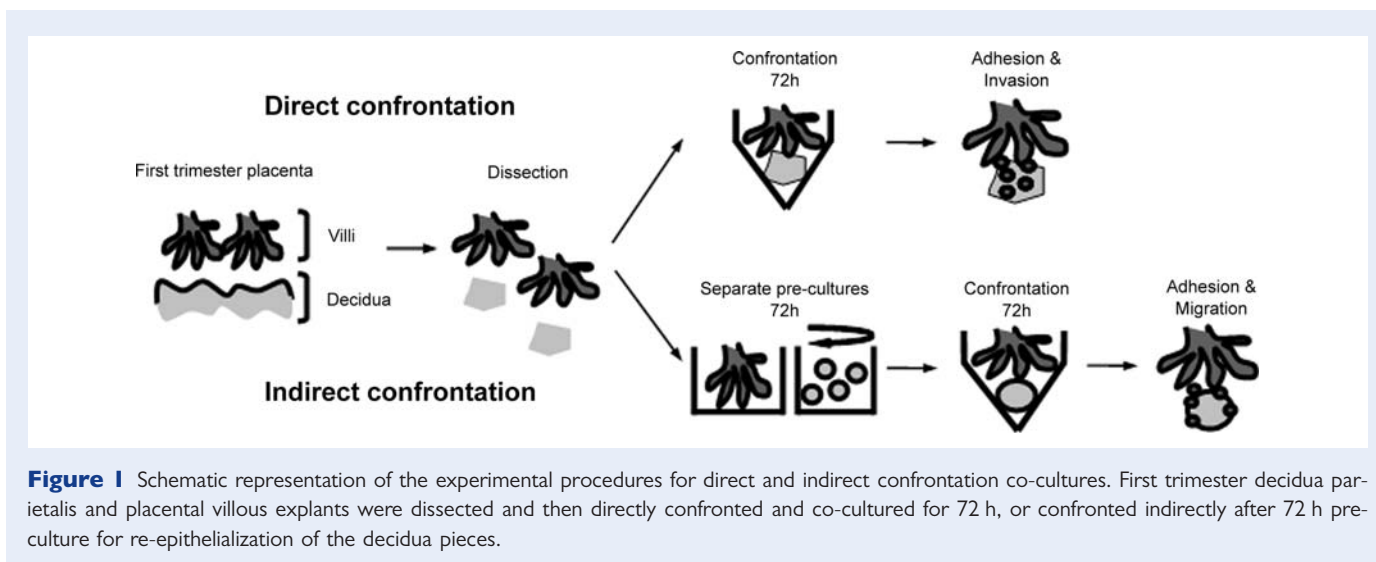
During indirect confrontation (with epithelium) trophoblast cells rather migrate along the surface of the decidua piece (Fig. 2E). Re-epithelialization within 2–3 days was previously described in similar model systems (Vicovac *et al.*, 1995; Helige *et al.*, 2001) and could be confirmed in our model systems: a cross section of a decidua piece cultured for 50 h shows the beginning of outgrowth of cytokeratin 7 positive epithelial cells from a gland opening (Fig. 2F). The replenished epithelium after pre-culture of the decidua is built in the same way as the epithelium *in situ*: this is shown by immunofluorescent double staining with antibodies against cytokeratin 7 and entactin on sections of a cultured decidua piece (Fig. 2G) as well as of uncultivated decidua (Fig. 2H). In both cases, cytokeratin 7 positive epithelial cells are situated on the top of the entactin positive basement membrane. The replenished epithelium may not exactly represent the luminal epithelium, but similar to the *in vivo* situation, it consists of a basement membrane and epithelial cells lying on the top of it. Thus, it may well serve as a model for uterine epithelium.

### EVTs and uterine glands

#### *In situ*

Invasion of EVT into the decidua basalis was visualized by immunostaining of paraffin sections of first trimester placental tissues with the anti-HLA-G antibody 4H84. EVT can be found in the decidual stroma and close to uterine glands (Fig. 3A). EVT come into close contact with the glandular epithelium and seem to penetrate the basement membrane (Fig. 3B), finally replacing glandular epithelial cells (Fig. 3C).

EVT can be found in close proximity to, as well as inside, the lumen of uterine glands throughout the first trimester (Fig. 4). Paraffin sections of invaded first trimester decidua basalis were immunodoublestained with antibodies against HLA-G and cytokeratin 7. Figure 4 shows that EVT are situated nearby uterine glands and replace glandular epithelial cells during the gestational period of 6–11 weeks of pregnancy (Fig. 4C–F, H, I and K). In some cases, EVT even infiltrate the uterine lumen (Fig. 4B and G). In every first trimester decidual section that was doublestained for HLA-G and cytokeratin 7, EVT were found in and/or in very close proximity to uterine glands. In the invaded parts of sections of decidua basalis,



**Figure 1** Schematic representation of the experimental procedures for direct and indirect confrontation co-cultures. First trimester decidua parietalis and placental villous explants were dissected and then directly confronted and co-cultured for 72 h, or confronted indirectly after 72 h pre-culture for re-epithelialization of the decidua pieces.

55 ± 7% (mean ± SEM;  $n = 10$ , range 6–11 weeks) of glandular cross sections were associated with or infiltrated by EVT.

#### *In vitro*

In our model systems invasive EVTs can be found in close proximity to uterine glands (Fig. 5). Immunofluorescent double staining using antibodies against HLA-G to detect EVT (red–yellow) and cytokeratin to detect trophoblasts and glandular epithelial cells (yellow–green) was applied on sections from direct and indirect confrontations (Fig. 5B and C). During indirect confrontation, EVTs migrate along the surface of the re-epithelialized decidua piece and towards the glandular lumen (Fig. 5B). During direct confrontation, EVTs seem to invade the decidual interstitium towards the glands, and single trophoblast cells can even be found integrated into the layer of the glandular epithelium (Fig. 5C, circle). The localization and putative movement of EVTs was visualized in schematic drawings (Fig. 5D and E).

Staining of additional direct confrontations showed EVTs lying between glandular epithelium and glandular basement membrane (Fig. 6). Double immunofluorescent staining for HLA-G and cytokeratin 7 revealed the presence of EVTs beneath glandular and uterine epithelial cells (Fig. 6C and D). Double immunofluorescence for the basement membrane marker entactin and HLA-G on serial sections demonstrated that EVTs were lying on the top of the basement membrane (luminal side; Fig. 6E and F).

## Discussion

Here, we present *in vitro* double tissue confrontation co-culture model systems of decidual tissues with villous explants to mimic EVT invasion during placentation. We are able to show infiltration of EVTs into the lining of the glandular epithelium *in situ* and *in vitro*, thus opening the way for glandular secretion products to reach the intervillous space.

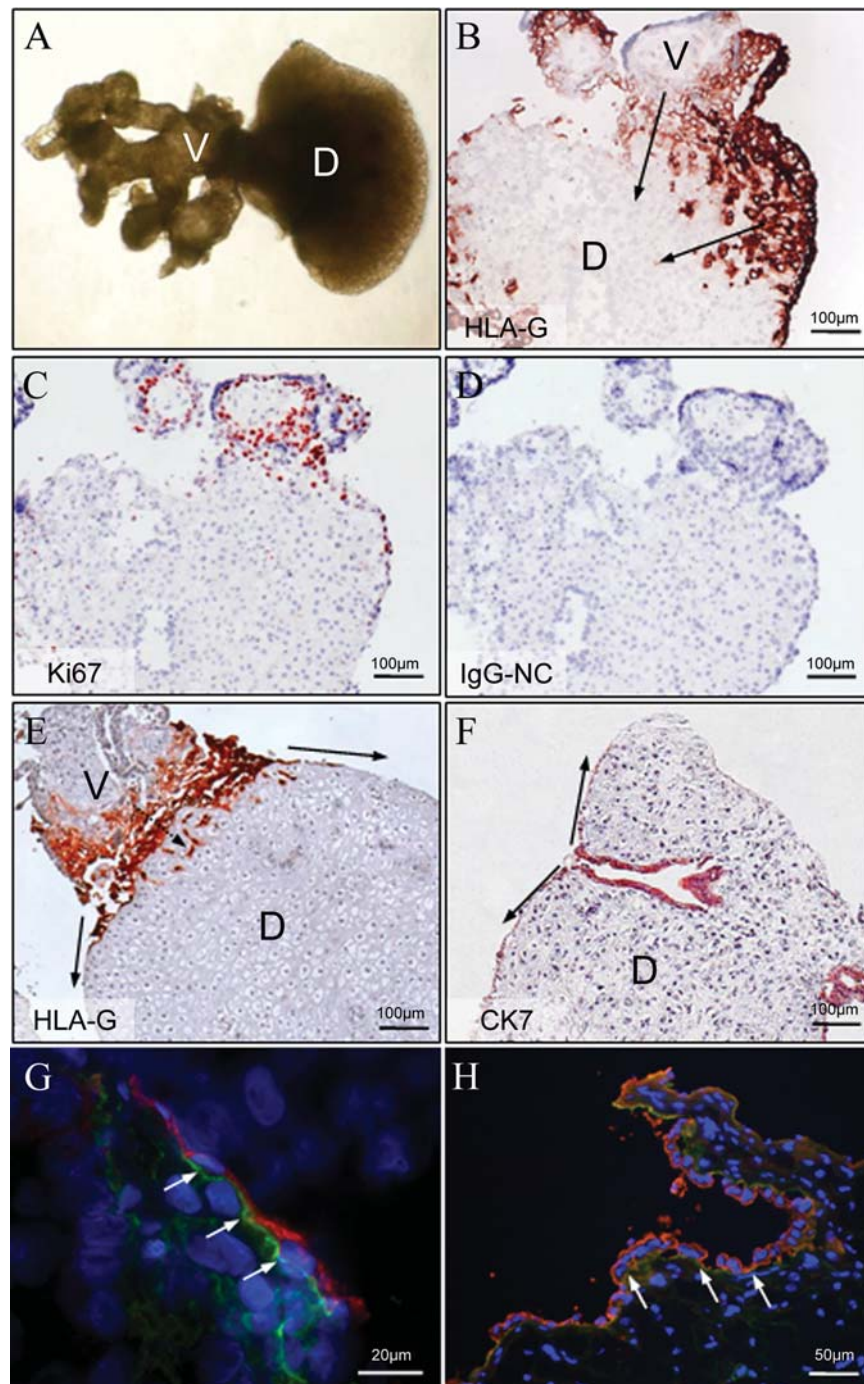
Such *in vitro* model systems represent useful tools for studying trophoblast invasion although each artificial system has certain limitations. The main advantage of using whole tissue specimens is that the cells of interest remain in their appropriate cellular as well as extracellular environment and thus these systems mostly maintain the context of

the *in vivo* situation. At the same time the major disadvantage of such systems is that manipulation of specific cells is nearly impossible without affecting other cell types as well.

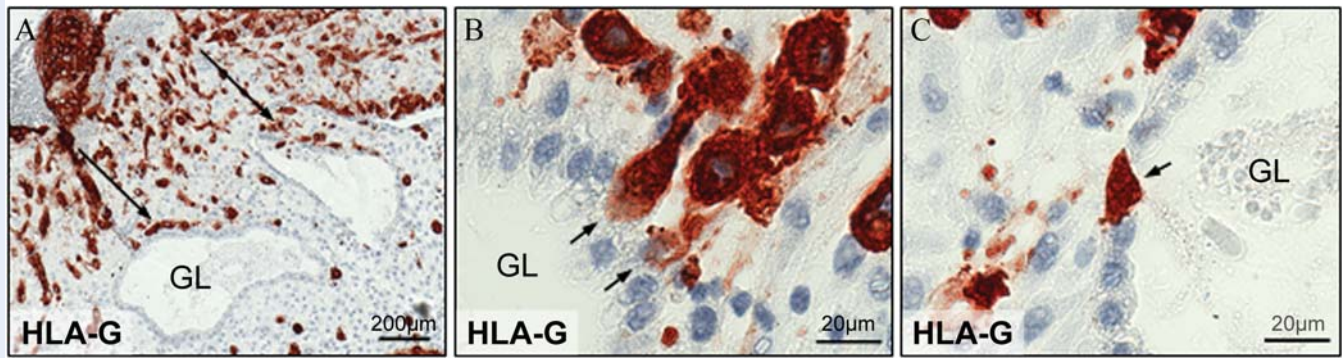
Placental explant cultures have been proven to be a useful model for studying proliferation, growth and differentiation as well as invasion (Miller et al., 2005). Various model systems have been developed over the last 15 years. Villous explants have been cultured on extracellular matrices such as collagen I (Lacey et al., 2002), extracellular matrix (Laminin) from Engelbreth–Holm–Swarm murine sarcoma (Seeho et al., 2008) and Matrigel (Genbacev et al., 1993) as well as on decidua parietalis (Vicovac et al., 1995; Babawale et al., 2002; Dunk et al., 2003).

Placing villous explants on collagen I allows us to evaluate processes of trophoblast migration. Monitoring of invasion into an extracellular matrix is possible with matrices such as Matrigel. More complex investigations, such as assessing events of cellular interactions or vascular remodelling, can only be achieved using double tissue co-cultures.

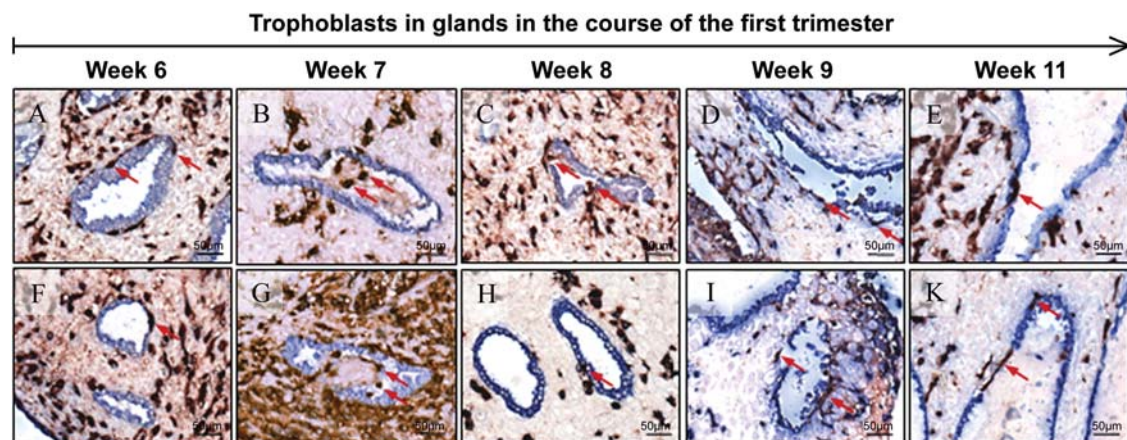
In other co-culture model systems, decidual and villous tissues were glued together by embedding in Matrigel/rat tail collagen with the elegant possibility to orientate the tissues (epithelial side/luminal side of decidua towards trophoblasts) before and during co-culture (Vicovac et al., 1995; Dunk et al., 2003). However, Matrigel as well as other extracellular matrices are complex mixtures with various unknown components, which may well influence the actions and interactions of cells and tissues. We minimize the presence of factors possibly influencing trophoblast invasion in our confrontation co-culture system. In the reaction tubes, tissues simply float together in the culture medium without further influence of growth factors. Furthermore, handling of the tissue fragments is reduced, thus avoiding potential tissue damage. There is no possibility of tissue orientation—epithelial/luminal side of the decidua towards the villous tissue—however, the availability of direct confrontation (without epithelium) and indirect confrontation (with epithelium) allows comparison of invasion with/without epithelium. Supplementation of hormones to the culture medium of invasion assays has already been performed (Helige et al., 2001, 2008), but to the best of our knowledge hormones have never been supplied to decidual–villous co-cultures. Since the presence of progesterone and 17- $\beta$ -estradiol is crucial to



**Figure 2** Model systems for invasive trophoblasts. **(A)** Photograph of a confrontation co-culture, the villus (V) firmly attached to the decidua (D) after 72 h of confrontation. **(B)** HLA-G immunostaining of a section of a direct confrontation (GA 8 weeks) after 72 h: strong invasion of EVT into the decidua stroma can be seen. **(C)** Serial section to (B) immunostained with the proliferation marker anti-Ki67. **(D)** Isotype negative control (NC). **(E)** HLA-G immunostaining of a section of an indirect confrontation (with epithelium, GA 7 weeks), decidua was pre-cultured for 72 h prior to confrontation for re-epithelialization. A trophoblastic cell column has formed but invasion into the decidua stroma is only superficial. **(F)** Re-epithelialization of decidua (GA 8 weeks) after 50 h of culture: outgrowth of cytokeratin 7 (Ck 7) positive epithelial cells, arrows indicate putative growth direction of epithelial cells. **(G and H)** Immunofluorescent double staining with antibodies against Ck 7 to visualize epithelial cells (red) and entactin to visualize the basement membrane (green). The replenished epithelium (arrows) of cultured decidua (G) is similar to the epithelium of uncultivated decidua (H). Nuclei were counterstained with Hemalaun (A–F) or DAPI (G and H).



**Figure 3** EVT's reach and invade glands *in situ*. HLA-G immunostaining (antibody 4H84) of a paraffin section of decidua basalis (GA 11 weeks) invaded by EVT. **(A)** EVT's invade from cell columns towards uterine glands (arrows). **(B)** EVT cells (arrows) seem to penetrate the glandular basement membrane from the stromal side. **(C)** An EVT (arrow) has crossed the glandular basement membrane and has replaced a glandular epithelial cell. Nuclei were counterstained with Hemalaun. GL, glandular lumen.



**Figure 4** EVT's replace glandular cells during the first trimester. **(A–K)** Immunohistochemical double staining for HLA-G in red and Ck 7 in blue on paraffin sections of decidua basalis to visualize trophoblasts nearby and inside uterine glands. EVT's appear in dark violet, epithelial cells in blue. Red arrows indicate EVT's nearby and in uterine glands. **(A)** EVT's nearby uterine glands at week 6. **(C–F and H–K)** EVT's have replaced glandular epithelial cells. **(B and G)** EVT's have replaced uterine epithelial cells and have also infiltrated into the GL. From week 10, no specimen was available. No nuclear counterstain.

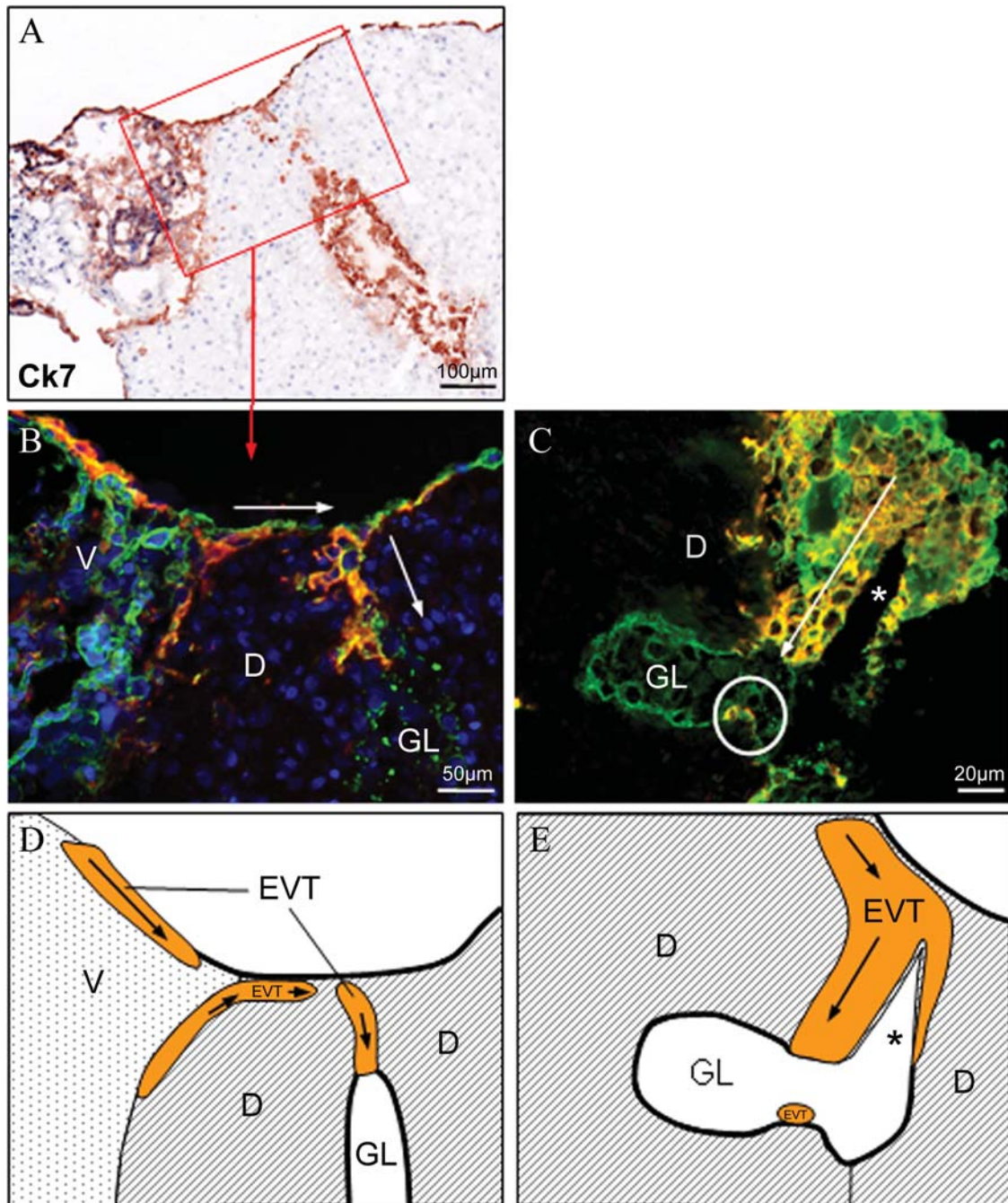
maintain decidual tissues, these hormones are present in our co-cultures to best mimic the *in vivo* situation. Similarly, the oxygen concentration is set to 20% oxygen (ambient air) since invading trophoblasts experience high oxygen concentrations different from villous trophoblast in the first trimester of pregnancy.

Re-epithelialization of cultured decidua pieces has already been described by other authors (Vicovac *et al.*, 1995; Helige *et al.*, 2001, 2008). Cells were observed to migrate out from the necks of endometrial glands on the top of the surface of the decidual stroma where they achieved a more flattened morphology (Vicovac *et al.*, 1995). Our cultured decidua pieces follow the same route of re-epithelialization (Fig. 2E).

We show that invasiveness of EVT's depends on the presence or absence of a decidual epithelium. In the absence of an epithelium, i.e. direct contact of trophoblasts and decidual stroma, deep invasion

into the decidual pieces is obvious (Fig. 2B). In indirect confrontation assays, when re-epithelialized decidua pieces are used for confrontation, EVT's migrate along the surface of the decidua pieces. Interestingly, the EVT's do not migrate on the top of the epithelium as assumed by Vicovac *et al.* (1995). Rather, it seems as if they migrate between epithelial cells and epithelial basement membrane (Fig. 6). This is true for the luminal epithelium as well as for the glandular epithelium. Such a localization of EVT's can also be seen *in situ* (Fig. 4A), where trophoblasts are located directly underneath the glandular epithelial cells. However, we do not have the proof that the trophoblasts are lying between epithelial cells and basement membrane here.

Our data also demonstrate that EVT's can be found nearby the glandular epithelium, *in situ* and *in vitro*. This finding is in accordance with data from Demir *et al.* (2002) showing cytokeratin positive EVT's in close proximity to decidual glands in placental tissues

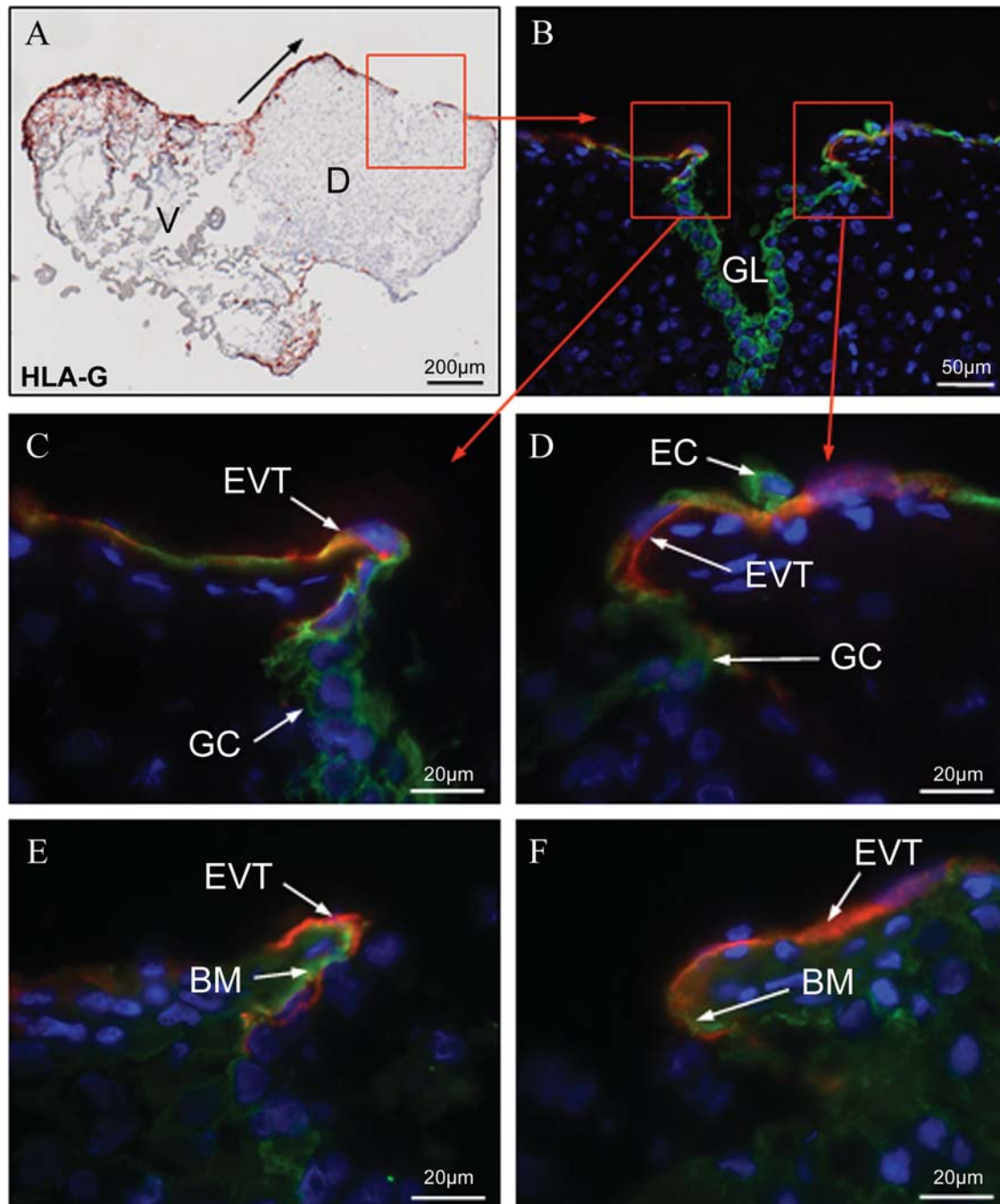


**Figure 5** EVTs are approaching uterine glands in direct and indirect confrontation assays. **(A)** Overview of an indirect confrontation (GA 6 weeks) with immunostaining against Ck 7 staining trophoblast and epithelium. **(B and C)** Immunofluorescent double staining using antibodies against HLA-G to visualize EVT (red–yellow) and Ck 7 to visualize trophoblasts as well as glandular and decidual epithelium (green). **(B)** During indirect confrontation trophoblasts migrate along the surface of the re-epithelialized decidua piece and towards the GL (marked by the white arrows). **(C)** During direct confrontation (GA 7 weeks), EVTs reach uterine glands after interstitial invasion through the decidual stroma (white arrow). Single cells are integrated into the layer of the glandular epithelium (circle). **(D and E)** Schematic representation of **(B)** and **(C)** to depict the different cell types and routes of invasion. Nuclei were counterstained with DAPI.

between Days 18 and 41 post-conception. Hempstock *et al.* performed studies on *in situ* specimens of early gestational material and showed that many invading trophoblasts, macrophages and natural killer cells were present in the stroma between the glands, but only

the latter two cell types were seen in close proximity to the glandular epithelium (Hempstock *et al.*, 2004).

To the best of our knowledge, the presence of EVTs lying inside glands has never been described in the literature. This may be



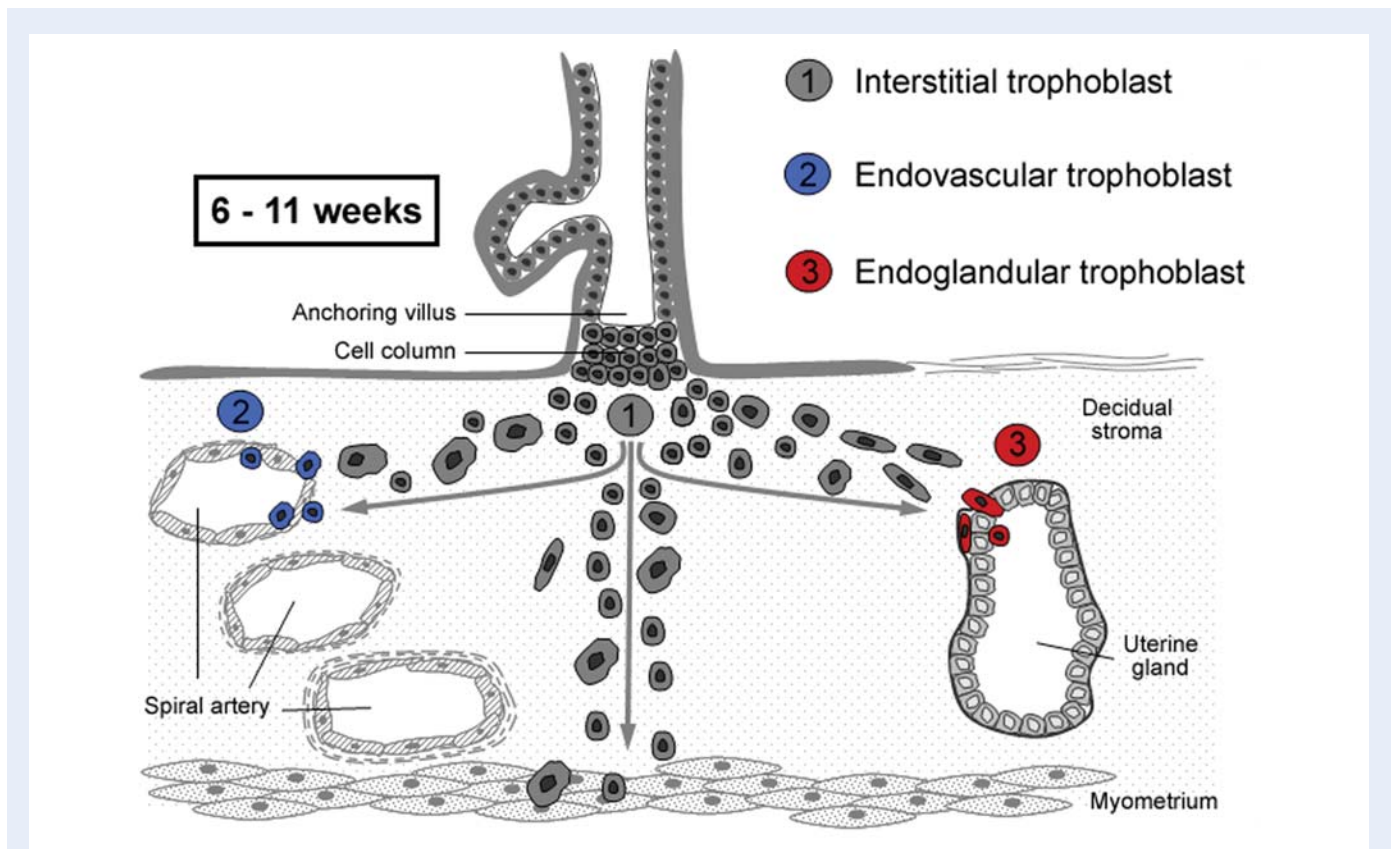
**Figure 6** Direct contact between EVT and glandular epithelial cells. **(A)** Overview of a direct confrontation (GA 7 weeks) with immunostaining against HLA-G. EVTs migrate from the villus along the surface of the decidua (black arrow). **(B–D)** Immunofluorescent double staining for Ck 7 and HLA-G shows glands and epithelium (green) and EVTs (red–yellow). Trophoblasts are located beyond glandular and uterine epithelial cells. **(E and F)** A serial section to the images in (B–D) labelled for HLA-G (red) and entactin to visualize the basement membrane (green). Trophoblasts are lying on the top of the basement membrane between the membrane and the glandular epithelial cells. Nuclei were counterstained with DAPI. BM, basement membrane; GC, glandular cell; EC, epithelial cell.

because earlier groups did not use specific antibodies to differentiate between EVTs and glandular epithelial cells. From our own experience from assessing haematoxylin and eosin sections as well as immunohistochemically stained sections only for cytokeratin 7, we can confirm that it is very difficult to identify trophoblasts at or in glands since

both cell types are stained with cytokeratin antibodies. Only using a double staining for cytokeratin and HLA-G clearly reveals the presence of EVT nearby, and even inside, glands.

It may well be that during implantation a glandular opening is already 'recruited' by the implanting blastocyst. However, looking at the





**Figure 7** Scheme of trophoblast invasion of the uterine glands. EVT's originate from cell columns of anchoring villi. During the first trimester of pregnancy EVT's invade through the decidual interstitium (1) and follow different routes of invasion. Interstitial trophoblasts invade up to the first third of the myometrium, thereby anchoring the placenta to the uterus. Endovascular trophoblasts (2) line and remodel spiral arteries, subsequently establishing the feto-maternal blood flow. Endoglandular trophoblasts (3) have reached the gland and replace single glandular epithelial cells thus coming into direct contact with the glandular lumen.

number of glands penetrated by trophoblast even at up to 11 weeks of gestation, other mechanisms need to come into play to explain the large number of glandular cross sections that have been found to contain EVT's.

Burton *et al.* (2002) showed that the secretions of the uterine glands are released in the intervillous space but provided no explanation on how the lumen of the uterine gland may be connected with the intervillous space. In contrast to earlier studies, we had the possibility to apply specific antibodies to clearly distinguish between invasive EVT and glandular cells. Our data indicate that EVT's originating from anchoring villi migrate through the decidual interstitium, break through the basement membrane of the epithelium, migrate between basement membrane and epithelial cells and sometimes even replace glandular epithelial cells. This new route of trophoblast invasion is schematically represented in Fig. 7. Clear evidence of EVT's even lying in the glandular lumen is demonstrated in Fig. 4B and C. According to the definition in Benirschke *et al.*, there are different phenotypes of invasive EVT's, the interstitial trophoblast with its subtypes (Kemp *et al.*, 2002) and endovascular trophoblast (Frank and Kaufmann, 2006).

Referring to our data, we suggest a new route of trophoblast invasion, the 'endoglandular trophoblast'. Endoglandular trophoblasts nearby uterine glands, replacing the glandular epithelium or even present in the glandular lumen, were found repeatedly and in every

specimen assessed throughout the first trimester of pregnancy. The series of images with endoglandular trophoblasts in Fig. 4, as well as the approach of counting endoglandular trophoblasts in glandular cross sections, indicate that invasion into glands is not a rare event. However, a simple quantification approach from single *in situ* sections without a clear orientation does not meet the requirements of a valid quantification approach. Hence, a further study including more and larger tissue samples is needed.

Invasion of spiral arteries is a directed and controlled process. Starting from interstitial trophoblast, a subset of EVT's directs themselves towards arteries which are invaded from the interstitial side and eventually the endovascular trophoblasts reach the lumen of the vessels after penetration of the vessel's media. Endoglandular invasion displays similar aspects to endovascular invasion: EVT's can be found in close contact and within the lumen of glands, sometimes replacing glandular epithelial cells. In general, not all luminal structures in the decidua are invaded by EVT's, e.g. uterine veins. Whether or not pathologies derive from failure of invading decidua glands has not been addressed yet in any study. However, it is tempting to speculate that early spontaneous miscarriages may result from missing nutrition following a failure of invasion of uterine glands.

The replacement of glandular cells by endoglandular trophoblasts displays a possible mechanism for opening and connection of the

uterine glands towards the intervillous space, resulting in histiotrophic nutrition of the embryo prior to onset of the maternal blood flow. With our novel confrontation co-culture model systems, we are able to imitate, at least partially, the changes in maternal tissues occurring during trophoblast invasion and placentation *in vivo*.

## Authors' roles

G.M. has performed all tissue culture experiments, G.M. and B.H. have written the manuscript, B.H. and M.G. supervised the experiments, K.O. and R.T. assisted in developing the culture method, A.G. obtained termination material and patient informed consent.

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