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human reproduction **ORIGINAL ARTICLE** ***Early pregnancy***

Evidence from the very beginning: endoglandular trophoblasts penetrate and replace uterine glands *in situ* and *in vitro*

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STUDY QUESTION: How is histiotrophic nutrition of the embryo secured during the first trimester of pregnancy?

SUMMARY ANSWER: Rather than specifically focusing on invasion into spiral arteries, extravillous trophoblasts also invade into uterine glands (endoglandular trophoblast) from the very beginning and open them toward the intervillous space.

WHAT IS KNOWN ALREADY: Extravillous trophoblasts can be found in close contact and within the lumen of uterine glands, sometimes replacing glandular epithelial cells.

STUDY DESIGN, SIZE, DURATION: As well as extensive screening of specimens from first trimester placentation sites *in situ* we used a previously established three-dimensional co-culture *in vitro* model system of first trimester villous explants with non-invaded decidua parietalis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: First trimester placentas were obtained from elective terminations of pregnancies ($n = 48$) at 5–11 weeks of gestational age. A subset was processed for confrontation co-culture ($n = 31$). Invaded decidua basalis was obtained from 20 placentas. All tissues were sectioned, subsequently immunostained and immunodoublestained with antibodies against keratin 7 (KRT7), major histocompatibility complex, class I, G (HLA-G), matrix metallopeptidase 9 (MMP9), von Willebrand factor (VWF) and the appropriate Immunoglobulin G (IgG) negative controls. Replacement of endothelial/epithelial cells by extravillous trophoblasts was quantified semi-quantitatively. Additionally, hematoxylin and eosin-stained archival specimens from early implantation sites were assessed.

MAIN RESULTS AND THE ROLE OF CHANCE: The earliest available specimen was from around Day 10 after conception; already at this stage trophoblasts had penetrated into uterine glands and had started to replace the epithelium of the glands. Endoglandular trophoblasts replaced uterine glands *in vitro* and *in situ* and could be found in the lumen of invaded glands. Quantitative analysis revealed significantly more replacement of epithelial cells in glands (63.8 ± 22.1%) compared with endothelial cells in vessels (26.4 ± 8.8%). Accumulated detached glandular epithelial cells could be repeatedly observed in the lumen of invaded glands. Additionally, in areas of trophoblast invasion the glandular epithelium seemed to be completely disintegrated compared with glandular epithelium in the non-invaded parts of the decidua. Whole tissue specimens were used *in vitro* and *in situ* instead of cell lines; these systems mostly maintain the context of the *in vivo* situation.

LIMITATIONS, REASONS FOR CAUTION: This is a descriptive study supported by *in vitro* experiments. However, a histological section will always only be a snapshot and quantification from histological sections has its limitations.

WIDER IMPLICATIONS OF THE FINDINGS: This study further strengthens the hypothesis of histiotrophic nutrition of the embryo prior to the establishment of the maternal blood flow toward the placenta. Invasion of uterine glands by endoglandular trophoblasts may have more impact on the outcome of early pregnancy than assumed up to now.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the Austrian Science Fund (grant P24739-B23 and P23859-B19), the Franz Lanyar Foundation of the Medical University of Graz (Project #347 and #358), the Post Doc program of the Medical University of Graz and by 'Land Steiermark' (grant A3-16.M-1/2012-32). None of the authors declares a conflict of interest.

Key words: placenta / endoglandular trophoblast / invasion / uterine gland / co-culture / implantation / model system

Introduction

Maternal blood flow toward the placenta for hemotrophic nutrition of the embryo is not established before the end of the first trimester (Hustin and Schaaps, 1987; Jaffe *et al.*, 1997; Kliman, 2000). Prior to that, the intervillous space is filled—besides plasma ultrafiltrated through plugs of endovascular trophoblasts—with secretion products primarily derived from uterine glands (Burton *et al.*, 2007). More than 10 years ago a related hypothesis stated that these secretion products are an important source of nutrients for the placenta and the embryo during the first trimester of pregnancy (histiotrophic nutrition) (Burton *et al.*, 2002). Recently, more evidence for this hypothesis has been collected. Decidual glandular epithelial cells contain large amounts of glycogen (Dockery *et al.*, 1988) which, according to Jones *et al.* (2015), is broken down to release diffusible sugar molecules. They deduced that first trimester histiotrophic molecules are internalized by the syncytiotrophoblast and then broken down there. This process declines with the start of hemotrophic nutrition (Jones *et al.*, 2015).

The knowledge about role and function of uterine glands during pregnancy has recently been reviewed by Spencer *et al.* (Spencer, 2014). The authors clearly state that research so far strongly supports the hypothesis that uterine glands and their secretion products play important biological roles in pregnancy. However, in humans there is a significant knowledge gap on how uterine glands and placental histiotrophic molecules support early pregnancy. Also, respective data on the impact of deficient glandular function on pregnancy success and complications are still missing. To date, only little is known about the detailed way in which uterine glands are opened toward the placental intervillous space. More information on how the secretion products are made accessible for the embryo is needed.

Due to ethical constraints it is not possible to investigate the respective processes during early pregnancy in humans *in vivo*. Hence, *in vitro* model systems for early placentation need to be used as tools to mimic the processes during early placentation. Questions concerning blood vessel remodeling (Dunk *et al.*, 2003), distribution of immune cells in the neighborhood of the trophoblast invasive front (Helige *et al.*, 2014), possible new routes of trophoblast invasion (Moser *et al.*, 2010) and others have been answered successfully with these model systems.

Invasion of extravillous trophoblasts (EVTs) into maternal tissues serves to attach the placenta to the uterus on the one hand and to support nutrition of the embryo on the other hand. A few years ago, we were able to demonstrate that similar to the invasion of spiral arteries, uterine glands are invaded by EVTs as well. Hence, we suggested a new route of trophoblast invasion, the so-called endoglandular trophoblast (Moser *et al.*, 2010). Endoglandular trophoblasts have been described as invading EVTs located in spatial proximity to uterine glands, attaching to their epithelium or already being present in the lumen of uterine glands (Fitzgerald *et al.*, 2010; Moser *et al.*, 2010; Huppertz *et al.*, 2014). It is tempting to speculate on their role in enabling histiotrophic nutrition of the embryo prior to the establishment of the uteroplacental circulation.

The aim of this study was to investigate the presence of endoglandular trophoblasts during very early placental development and to strengthen their putative role in enabling histiotrophic nutrition of the embryo. Besides extensive screening of specimens from first trimester placentation sites *in situ,* we used our previously established three-dimensional co-culture *in vitro* model system of first trimester villous explants with non-invaded decidua parietalis. This model system closely resembles the *in vivo* situation during early pregnancy.

Materials and Methods

Tissue collection

First trimester placentas were obtained from elective terminations of pregnancies (gestational age (GA) 5–11 weeks, $n = 48$). Invaded decidua basalis was obtained from 20 placentas with GA from 5 to 11 weeks. For the *in vitro* experiments only samples from placentas with GA of 6–9 weeks were used ($n = 31$). Informed consent was obtained from each

Table I Primary antibodies used in immunohistochemistry.

Antigen/antibody Clone/Cat No	Company	Dilution IHC cryo	IHC ffpe	Host/isotype
KRT7 (APO6204PU-N)	Acris (Herford, Germany)	–	1:200	Rabbit IgG pc
HLA-G (MEM-G/9)	Exbio (Prague, Czech Republic)	1:2000	–	Mouse IgG mc
HLA-G (4H84)	Exbio (Prague, Czech Republic)	1:5000	1:10000	Mouse IgG mc
VWF (F3520)	Sigma-Aldrich (St. Louis, USA)	1:1000	1:000	Rabbit IgG pc
MMP9 (#NB110-57222)	Eubio (Vienna, Austria)	–	1:50	Rabbit IgG mc
Mouse IgG1 (DAK-GO1)	Dako (Carpinteria, USA)	1:100	1:100	Mouse IgG mc
Rabbit Immunoglobulin Fraction (X 0903)	Dako (Carpinteria, USA)	1:300	1:300	Rabbit

mc, monoclonal; pc, polyclonal; IHC, immunohistochemistry; ffpe, formalin fixed paraffin embedded.

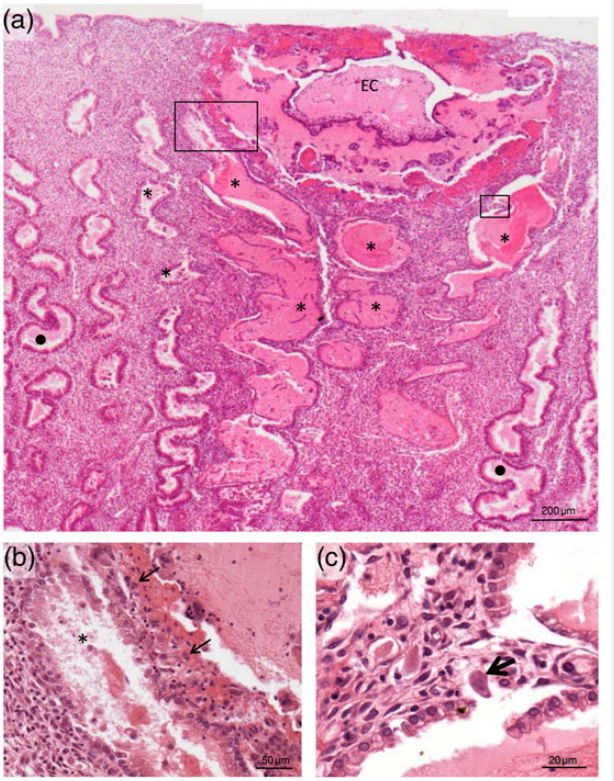

Figure 1 Trophoblasts penetrate uterine glands during very early pregnancy. Hematoxylin and eosin stained section of a human implantation site at Day 10 (archival specimen). (**a**) Overview: the early conceptus (EC) has already implanted into the decidual stroma beneath the uterine epithelium. Lumina of the uterine glands beneath the early conceptus seem expanded and filled with fluid (probably a mixture of glandular secretion products and blood). Glandular epithelia nearby the invading early conceptus (asterisk) seem to be disintegrated compared with glandular epithelium in the non-invaded parts of the decidua (black dots). (**b**) Detail of the left hand inset in (a); arrows show the direction of trophoblasts invading the decidua and penetrating the uterine gland. (**c**) Higher magnification of the right hand inset in (a); arrows show single trophoblasts penetrating the uterine gland.

woman with approval of the ethical committee of the Medical University of Graz. Tissues were rinsed in Hank's buffered salt solution (HBSS Gibco Life Technologies, Austria), supplemented with 1% v/v penicillin/streptomycin and 1% v/v amphotericin B (Gibco Life Technologies), placed in culture media (Dulbecco's modified Eagle medium (DMEM) high glucose with glucose with sodium pyruvate, without l-glutamine) supplemented with 10% v/v fetal calf serum (FCS), 2% v/v l-glutamine, 1% v/v penicillin/streptomycin, 1% v/v amphotericin B (Gibco Life Technologies), 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 decidua parietalis with intact epithelium were selected from the tissue samples and dissected in culture media. Absence of invasive trophoblast cells from the selected decidua parietalis was routinely checked by major histocompatibility complex, class I, G (HLA-G) immunohistochemistry. From every placenta various tissue samples were collected for fixation in 4% v/v paraformaldehyde and subsequent paraffin embedding. For the preparation of formalin fixed paraffin embedded (FFPE) sections, tissues were fixed in 4% v/v neutrally buffered formalin for at least 24 h and routinely embedded in paraffin. Additional samples were embedded in tissue freezing medium (Tissue Tek; Sakura Finetek Inc.; Torrance, USA) and stored at −80°C. Additionally, archival specimens (hematoxylin and eosin (H&E) stained slides) from the Institute of Cell Biology, Histology and Embryology, Medical University of Graz were assessed without details about origin and processing. These slides are normally used for student teaching.

Confrontation co-culture

Direct confrontation co-culture was performed as described previously (Moser *et al.*, 2010). Briefly, the non-invaded stroma of decidua parietalis was dissected in uniform, round-shaped pieces of ∼1 mm in diameter. Villous trees from the same placenta were cut into pieces of corresponding size (15–20 mg moist mass). One piece of decidua was co-cultured with one villous explant in 500 µl culture media in a 2 ml reaction tube (Eppendorf, Germany). Before culture the reaction tubes were perforated for gas-exchange. Villous tissue was confronted with decidual tissue from the same patient for 72 h at 37°C in a humified atmosphere of 95% v/v air −5% v/v CO_2. 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 (Tissue Tek), frozen at −80°C and processed for immunohistochemistry.

Preparation of sections

Serial 5 µm sections (cryo and paraffin) were cut and placed in duplicates on Superfrost Plus slides (Menzel, Braunschweig, Germany). Cryosections were air dried overnight and stored at −20°C. Every 10th slide of the confrontation co-cultures was routinely assessed using anti-HLA-G antibodies. For subsequent staining cryo-slides were thawed, air dried, fixed in acetone, air dried again for 5 min and rinsed in phosphate buffered saline (PBS). FFPE 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 (Leica Biosystems, Nussloch, Germany) in a pressure cooker (Model DC2002, Biocare Medical, Concord, USA) for 7 min at 120°C before immunohistochemistry.

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, Vienna). Table I 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 IgG fractions as isotype controls (Table I).

Immunohistochemical double staining

Immunohistochemical double labeling was performed using the Multivision Polymer Detection system (MultiVision anti-rabbit/AP + anti-mouse/HRP polymers; Thermo scientific, Fremont, USA) according to the manufacturer's instructions. For cryosections, Ultra V Block was supplemented with 10% human AB-serum.

Quantification of trophoblast invasion

For quantification of trophoblast invasion serial sections were double-stained with antibodies against keratin 7 (KRT7)/HLA-G and Willebrand factor (VWF)/HLA-G. A microscope (model DM6000B; Leica) equipped with a motorized stage and a digital camera (model DP72; Olympus Austria GmbH, Vienna, Austria) was used for acquisition of 10 images per slide. Within the serial sections the same image section was selected. Images

were obtained from three different placentas (GA 4, 5 and 6 weeks) and were evaluated with the Zeiss AxioVision software version 4.8.2.0 (Carl Zeiss, GmbH, Vienna, Austria). In every image luminal cross sections of uterine glands or vessels were classified and counted as follows: non-invaded gland; gland with EVTs attached; gland with epithelial cell(s) replaced by EVTs and/or EVTs located in the lumen; non-invaded vessel; vessel with EVTs attached; vessel with endothelial cell(s) replaced and/or EVTs located in the lumen.

Statistical analysis

Data are reported as means ± standard deviations. Student's *t*-test was applied for the quantification of EVT replacing epi-/endothelium and EVT in spatial proximity between glands and vessels, after testing for normal distribution (Kolmogorov–Smirnov test). Statistical analysis was done using SPSS IBM Statistics 21. A *P*-value <0.05 was considered as significant.

Results

Trophoblasts penetrate uterine glands in the very beginning of pregnancy

Archival specimens were inspected and searched for the existence and detailed localization of trophoblasts associated with uterine glands. Archival H&E stained sections of the early implantation site and the subsequent invasive front were available. The morphology of invading trophoblasts differs from the morphology of other cell types in the event of implantation. A histologist well experienced with placenta development may identify the cells of interest without immunohistochemical staining. The main characteristic of invading trophoblasts is the larger and round shaped nucleus, compared with the smaller and more compact nuclei of the surrounding decidual cells. The epithelium of uterine glands is composed of a single row of mononuclear columnar cells. Respective images are presented in Figs 1 and 2.

Trophoblasts associated with uterine glands occur from the very beginning of pregnancy. The earliest available specimen was from around Day 10 after conception (Fig. 1). Already at this early stage of pregnancy trophoblasts are invading the decidua, penetrating the uterine glands and start to replace the epithelium of uterine glands. Beyond that the lumina of uterine glands beneath the early conceptus appear expanded and filled with secretion fluid. Further evidence for trophoblasts associated with uterine glands occurring in the very beginning of pregnancy was also observed in another specimen of human implantation site at GA 3 weeks (Fig. 2). Also at this stage of pregnancy single trophoblasts penetrate a uterine gland (Fig. 2b).

Endoglandular trophoblasts *in situ* and *in vitro*

Invasion of EVTs was visualized by immuno-double staining with the anti-HLA-G antibody 4H84 and an antibody against KRT7 ('trophoblasts and glands') (Moser *et al.*, 2011). We previously reported that about 55% of glandular cross sections (GA 6-11 weeks) were associated with or infiltrated by endoglandular trophoblasts (Moser *et al.*, 2010). More evidence for their appearance is shown in Figs 3–6. Additionally, immuno-double staining with an anti-HLA-G antibody and an antibody against VWF has been performed ('trophoblasts and vessels'). VWF serves as a marker for endothelial cells and also reacts with fibrinoid and erythrocytes. Since EVTs express both HLA-G and KRT7 they appear dark brown in the immuno-double staining 'trophoblasts and glands', compared with a lighter brown appearance in the immuno-double staining 'trophoblasts and vessels'.

Endoglandular trophoblasts migrate from trophoblastic cell columns through the decidual stroma (referred to as interstitial trophoblasts), finally reaching and infiltrating uterine glands (referred to as endoglandular trophoblasts) (Fig. 3a and b). They replace the glandular epithelium and thereby open the lumen of the uterine glands toward the intervillous space (Fig. 3c and d). Immunohistochemical double staining allows an easy identification of endoglandular trophoblasts in sections from invaded decidua basalis. Additionally an archival H&E specimen contained one of the rare cross sections through a trophoblastic cell column attached to the decidua with a uterine gland is opened toward the intervillous space, the uterine gland is partly replaced by endoglandular trophoblasts (Fig. 3e and f).

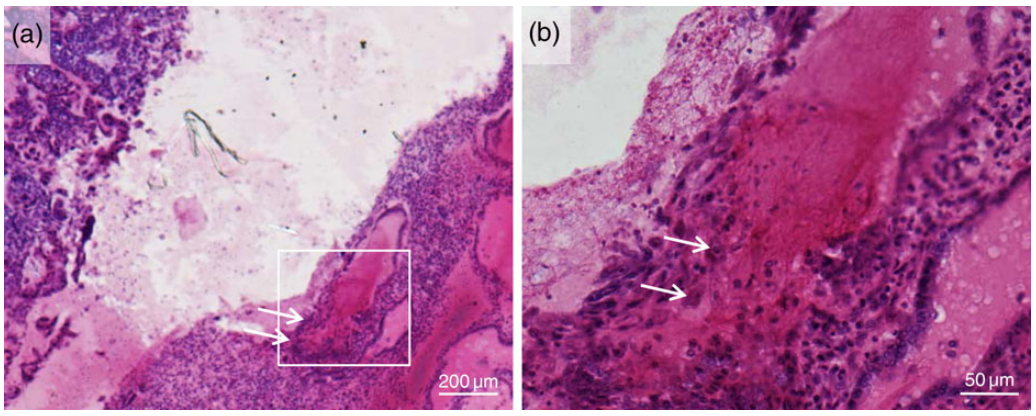

Figure 2 Trophoblasts penetrate uterine glands during very early pregnancy. Hematoxylin and eosin stained section of a human implantation site at gestational age 3 weeks (archival specimen). (**a**) Overview: The villous part has been disrupted from the decidua. In the broader overview under the microscope villi (V) and decidua (D) are clearly allocated (not shown). Arrows show the direction of trophoblasts invading the decidua and penetrating a uterine gland. (**b**) Higher magnification of the inset in (a); arrows show single trophoblasts penetrating a uterine gland.

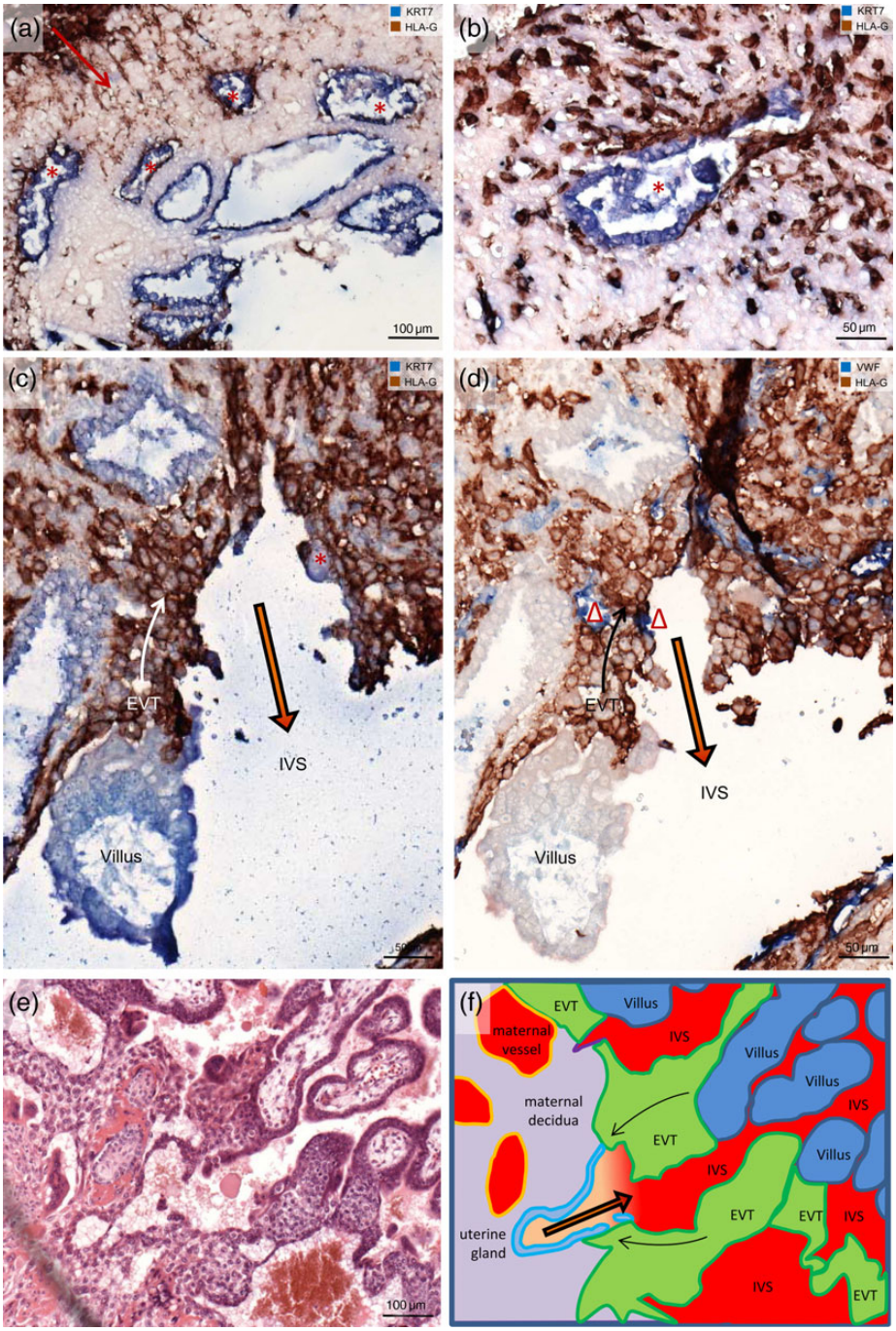

Figure 3 Endoglandular trophoblasts invade and replace the epithelium of uterine glands and open the glands toward the intervillous space *in situ*. Immunohistochemical double staining of invaded decidua (gestational age 6–12 weeks) for (**a**–**c**) keratin 7 (KRT7) (blue, serves here as marker for glandular epithelial cells) and major histocompatibility complex, class I, G (HLA-G) (appears dark brown, serves here as marker for extravillous trophoblasts (EVT)) besides (**d**) for von Willebrand factor (VWF) (blue, serves here as marker for vascular endothelial cells) and HLA-G (brown). Note that there is no nuclear counterstain. (a–b) EVT (brown) invade into the decidua (arrow) and replace the epithelium (blue) of uterine glands (asterisk). (c–d) Serial sections show a villus attached to the decidua via a trophoblastic cell column, EVTs (brown) invade the decidual stroma and replace the glandular epithelium, the lumen of the gland is opened toward the intervillous space; arrows show the putative flow direction of glandular secretions toward the intervillous space (IVS). Asterisk in (c) marks residual glandular epithelial cells, triangle in (d) marks residual blood vessel (blue). (**e**) Hematoxylin and eosin staining of the invasive front during the first trimester (archival specimen) and (**f**) corresponding schematic drawing shows a similar picture like in (c and d). Trophoblasts originating from trophoblastic cell columns (green) invade into decidual tissues (light purple). The epithelium of the uterine gland seems to have been partly replaced by endoglandular trophoblasts, on the decidual side there is still residual glandular epithelium (light blue). Thus, the lumen of the gland is opened toward the intervillous space (red). This enables the release of glandular secretion products into the intervillous space (arrow).

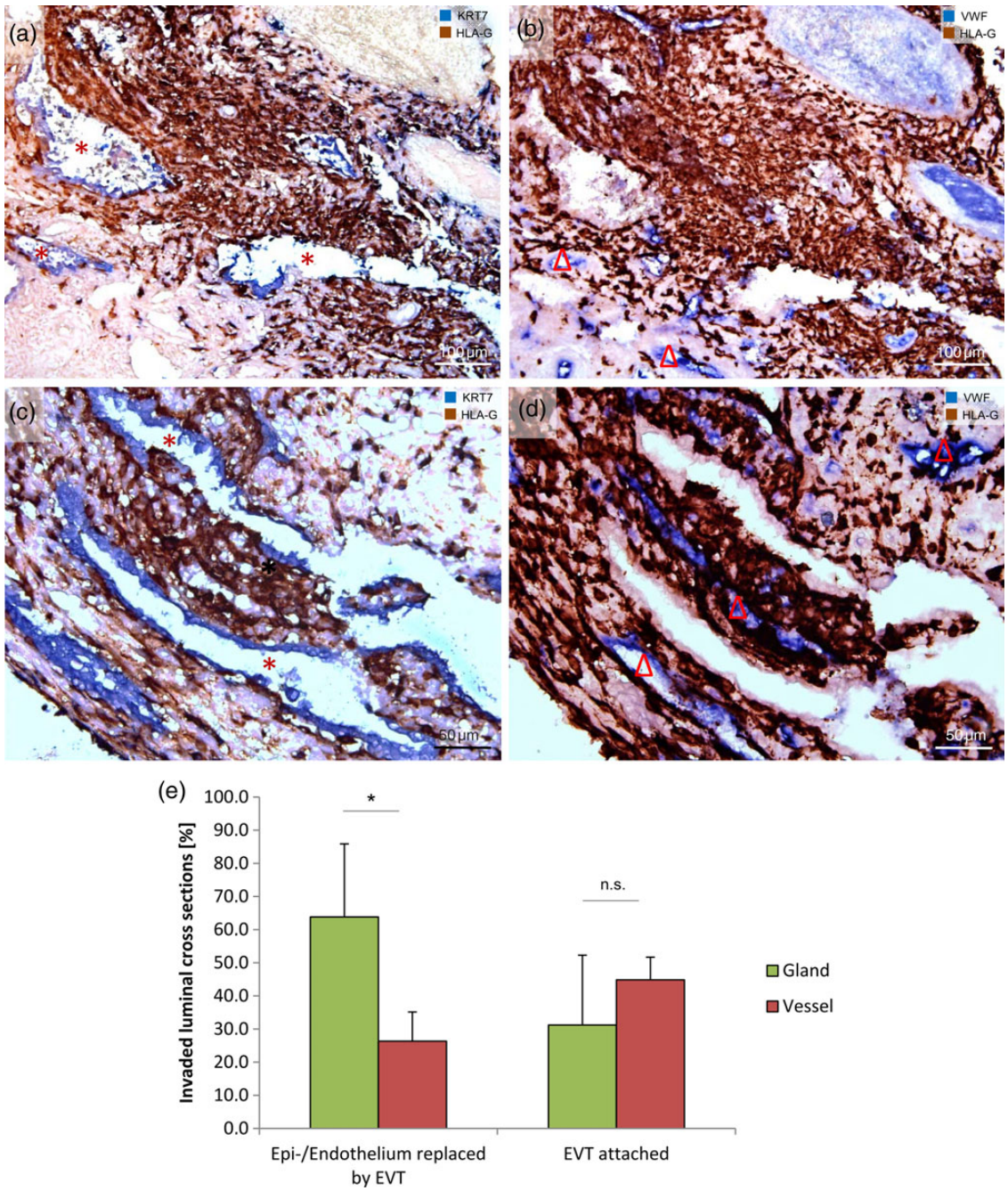

Figure 4 Distinction between endoglandular and endovascular trophoblasts. Invaded decidua with immuno-double staining (gestational age 6–8 weeks). Rows are composed of serial sections (**a**–**b**, **c**–**d**), sections in the left column are immuno-double stained for keratin 7 (KRT7) (blue, serves here as marker for glandular epithelial cells) and major histocompatibility complex, class I, G (HLA-G) (appears dark brown, serves here as marker for extravillous trophoblasts (EVTs), in the right column for von Willebrand factor (VWF) (blue, serves here as marker for vascular endothelial cells) and HLA-G (brown). Only immunohistochemical double staining enables clear identification whether the invaded structure was of glandular (asterisk) or vascular (triangle) nature. No nuclear counterstain. (**e**) Quantitative analysis reveals significantly more replacement of epithelial cells in glands (63.8 ± 22.1%) compared with endothelial cells in vessels (26.4 ± 8.8%). Attachment of interstitial trophoblasts to glands or vessels was not significantly different.

The comparison of serial sections with the respective specific immuno-double staining enables a clear identification whether the invaded structure was of glandular or vascular nature and allows thereby a distinction between endoglandular and endovascular trophoblast invasion (Figs 4 and 5). Quantitative analysis revealed significantly more replacement of epithelial cells in glands (63.8 ± 22.1%) compared with endothelial cells in blood vessels (26.4 ± 8.8%). In general, there are no significant differences in EVT attachment to luminal structures between glands and vessels (Fig. 4e).

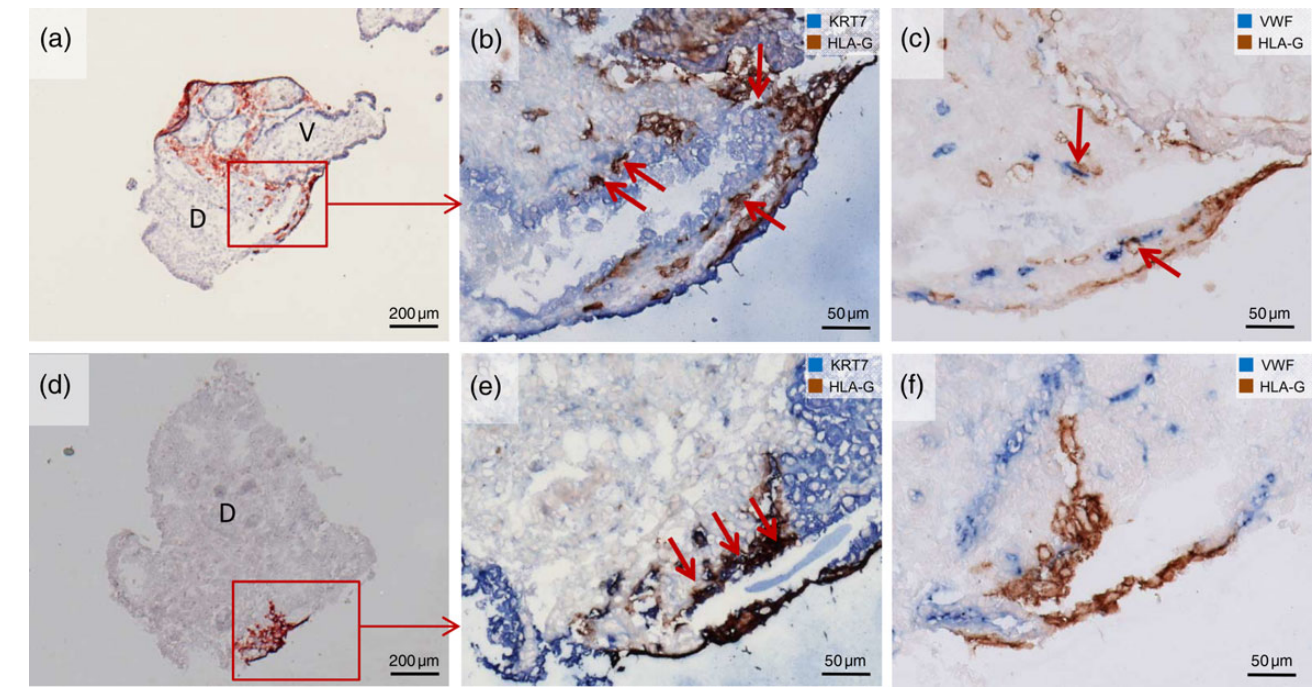

Figure 5 Endoglandular trophoblasts invade and replace uterine glands *in vitro* (gestational age 6–12 weeks). Each row is composed of serial sections (**a**–**c**, **d**–**f**), sections in the left column are immuno-stained for major histocompatibility complex, class I, G (HLA-G), in the middle column immuno-double stained for keratin 7 (KRT7) (blue, serves here as marker for glandular epithelial cells) and HLA-G (appears dark brown, serves here as marker for extravillous trophoblasts (EVTs)), and in the right column immuno-double stained for von Willebrand factor (VWF) (blue, serves here as marker for vascular endothelial cells) and HLA-G (brown). (a and d) Overview of confrontations. V, Villi, D, Decidua. (b and e) Glandular epithelial cells appear blue. Extravillous trophoblasts (arrows) invade into decidual tissues, reach the uterine glands and replace uterine glandular epithelial cells as endoglandular trophoblasts. (c and f) Vascular cells appear blue. Immunohistochemical double staining clearly shows the glandular/vascular nature of the invaded structure. Nuclei were counterstained with Hemalaun (a and d) or no nuclear counterstain was used (b,c,e and f).

In vitro pieces of non-invaded decidua parietalis and placental villi have been co-cultured and subsequently been analyzed for the presence of endoglandular trophoblasts. Equally to the situation *in situ*, endoglandular trophoblasts were found repeatedly also replacing uterine glands *in vitro* (Fig. 5b and e). Immuno-doublestaining ('trophoblasts and vessels') of serial sections confirmed the endoglandular localization of the cells (Fig. 5f) and also revealed the presence of endovascular trophoblasts in our model system for trophoblast invasion (Fig. 5c). Control tissues of each confrontation co-culture were routinely immunostained with antibodies against HLA-G and KRT7 and checked for the absence of invading trophoblasts both before and after culture. Isotype negative control antibodies did not reveal any staining (not shown).

Endoglandular trophoblasts: peculiarities

In most cases a specific immuno-(double) staining enables identification of whether the invaded structure was of glandular or vascular nature. Besides the already described occurrence of endoglandular trophoblasts situated nearby uterine glands, replacing the glandular epithelium or infiltrating the uterine lumen, two peculiarities have been observed when comparing serial sections. First, a partly invaded uterine gland is opened toward the lumen of a partly invaded uteroplacental vessel (Figs 3c, d and 6a, b). Second, in areas of trophoblast invasion accumulated detached glandular epithelial cells can be repeatedly observed in the glandular lumen (Fig. 6c). Glandular epithelium in these invaded areas seems to be completely disintegrated compared with the glandular epithelium in the non-invaded parts of the decidua. In the latter, glandular epithelium appears compact and well proportioned, no detached glandular epithelial cells can be seen in the glandular lumen (Fig. 6d). This was observed *in situ* and *in vitro*. Additionally, trophoblasts surrounding uterine glands are positive for matrix metallopeptidase 9 (MMP9) (Fig. 6f).

Discussion

Our data show that uterine glands are penetrated and invaded by trophoblasts from the very beginning of pregnancy onwards. Endoglandular trophoblasts invade uterine glands, replace their epithelium and open the glands toward the intervillous space *in situ* and *in vitro*. Distinctive features of endoglandular trophoblasts may further explain their function during placental development.

Already in 1959 J.D. Boyd described that the walls of glands nearby the implantation site are eroded in the implantation site of a 28-somite human embryo (this equates to GA 6–7 weeks). He also stated that the epithelium of the uterine glands frequently disappears when the trophoblast comes into contact with it, and the trophoblast may actually invade the lumen of the gland (Boyd, 1959). Later on Pijnenborg *et al.* (1980) described cytotrophoblasts and multinuclear giant cells in close association with a gland at the base of the decidua (Pijnenborg *et al.*,

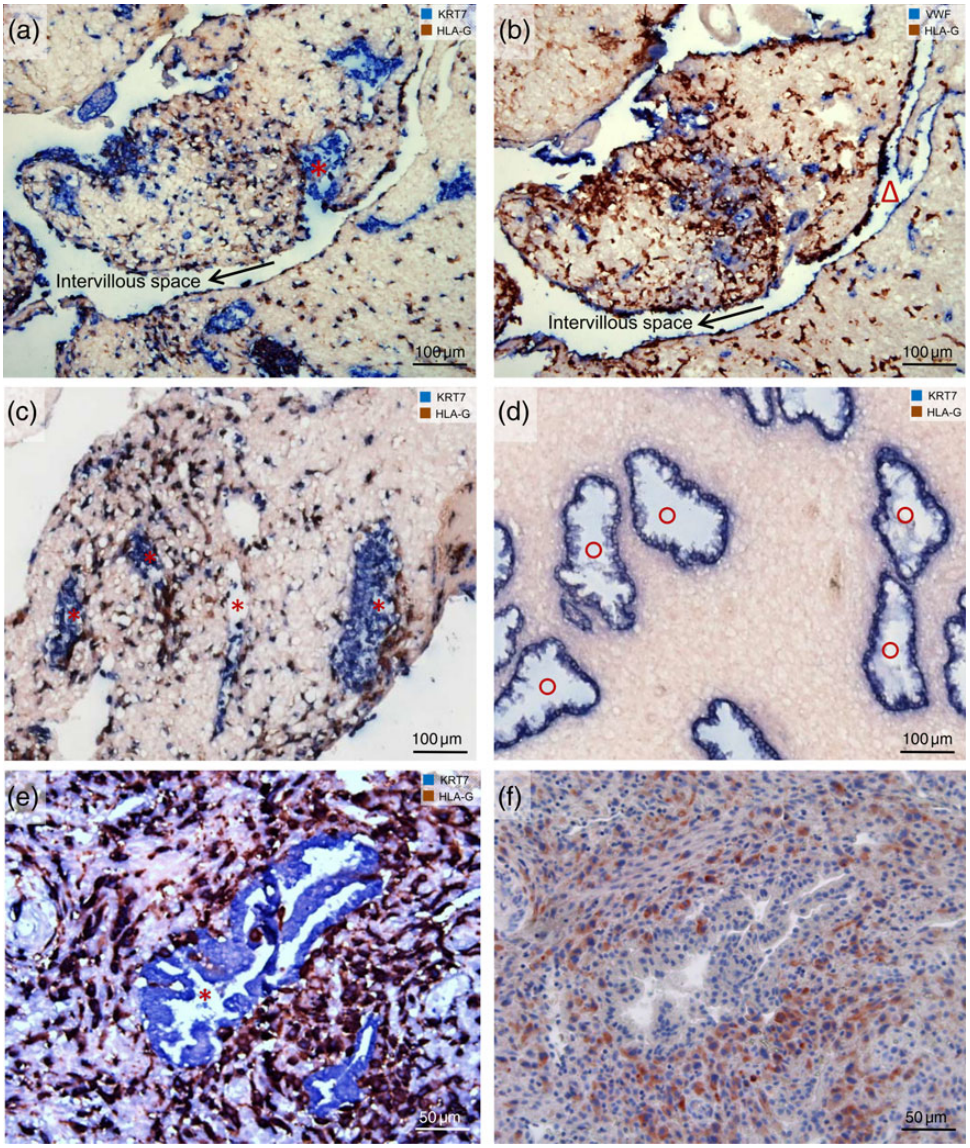

Figure 6 Endoglandular trophoblasts—peculiarities. Invaded decidua with immunohistochemical staining/double staining (gestational age week 5). Sections (**a**, **c**–**e**) are immuno-double stained for keratin 7 (KRT7) (blue, serves here as marker for glandular epithelial cells) and major histocompatibility complex, class I, G (HLA-G) (appears dark brown, serves here as marker for EVTs), section (**b**) is immuno-double stained for von Willebrand factor VWF (blue, serves here as marker for vascular endothelial cells) and HLA-G (brown), section (**f**) is immunostained for matrix metalloproteinase 9 (MMP9). (**I**) *Trophoblastic equivocality*: (a,b) Serial sections show a uterine gland, opened toward the lumen of an invaded vessel. Asterisk in (a) marks a uterine gland, where the epithelium is penetrated and partly replaced by endoglandular trophoblasts. The glandular epithelium is disintegrated. Triangle in (b) marks a uterine vessel, where the endothelium is partly replaced by endovascular trophoblasts. The arrow marks the presumable direction of glandular secretion/vascular plasma flow toward the intervillous space. (**2**) *Disintegrated versus intact glandular epithelium in invaded versus non-invaded regions*: (c) In the decidua basalis, virtually all glands are invaded by endoglandular trophoblasts. Glandular epithelia (asterisk) in the invaded parts appear disintegrated in comparison to (d) the non-invaded decidua parietalis from the same uterus. Here, the glandular epithelium (circle) appears compact and well-proportioned. (e–f) Serial sections, double staining shows an invaded gland (e), the epithelium is disintegrated, trophoblasts surrounding the uterine gland are positive for MMP9 (f). No nuclear counterstain (a–e) or nuclei were counterstained with Hemalaun (f).

1980). In accordance to our data the excellent and comprehensive image collection of Allen C. Enders (http://www.trophoblast.cam.ac.uk/info/enders.shtml) shows trophoblasts apparently penetrating uterine glands from the very beginning. There, trophoblasts invaded the underlying endometrial glands through the basement membrane of the uterine epithelium at stage 5a, i.e. 1 day after initiation of implantation. Also later trophoblasts apparently penetrate uterine glands. Allen Enders describes invasion of trophoblasts into uterine glands several times in his collection. It would be of great value to stain serial sections of such early implantation sites with antibodies against various markers to clarify whether the uterine glands literally prepare themselves to fulfill their future function in nourishing the embryo. Besides markers such as those for EVTs,

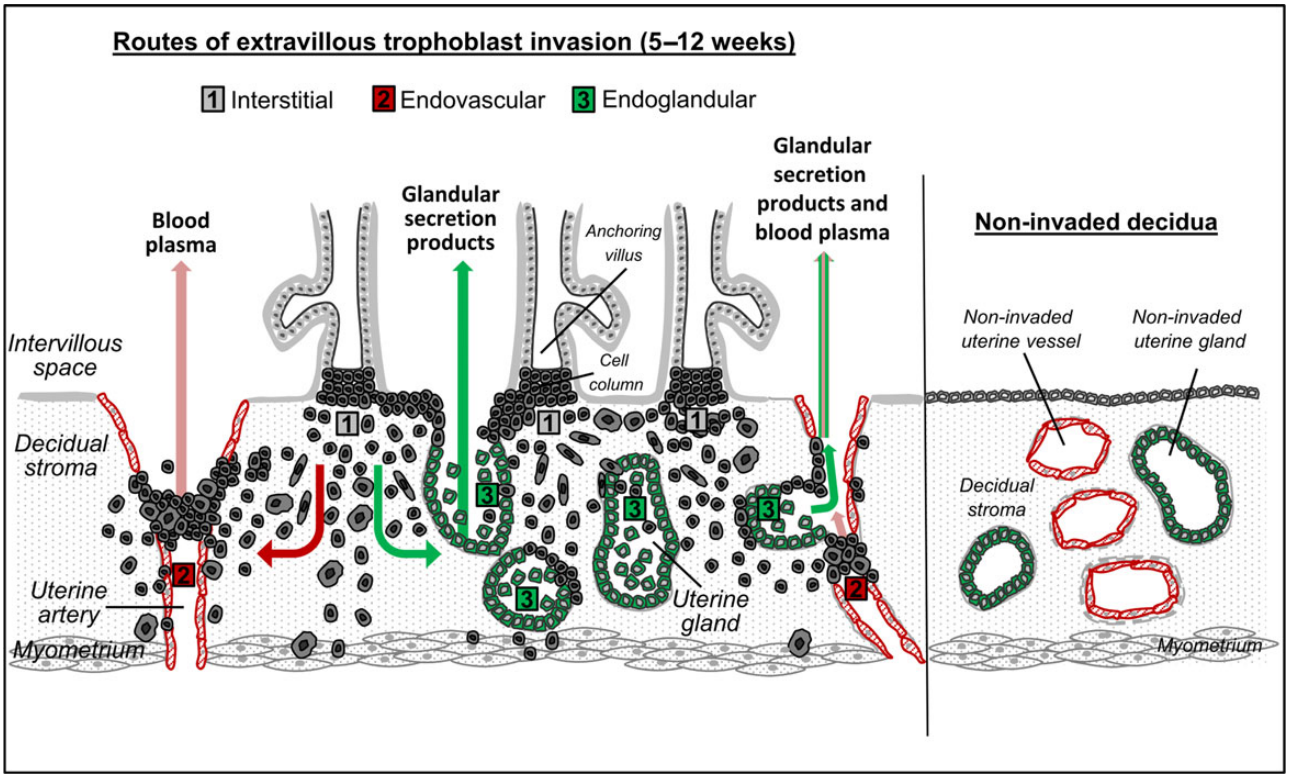

Figure 7 Scheme of first trimester human placenta and its nutritional supply. Extravillous trophoblasts originate from cell columns of anchoring villi. During the first trimester of pregnancy extravillous trophoblasts invade into the decidual interstitium (1) to the first third of the myometrium, thereby anchoring the placenta to the uterus. They also follow the (2) endovascular route of invasion, plug, line and remodel spiral arteries, thus being responsible for the establishment of the feto-maternal blood flow starting with the beginning of the second trimester. Prior to the opening of spiral arteries toward the intervillous space maternal blood plasma is seeping through the trophoblastic plugs. Endoglandular trophoblasts (3) are defined as trophoblasts situated nearby uterine glands, replacing the glandular epithelium or trophoblasts present in the glandular lumen. Additionally, endoglandular trophoblasts may open uterine glands toward the lumen of an invaded spiral artery, secretion products of the uterine gland may thereby reach the intervillous space together with the blood plasma seeping through the trophoblast plug of the spiral arteries. Glandular secretion products in the intervillous space enable histiotrophic nutrition of the embryo prior to the establishment of the feto-maternal blood flow, i.e. hemotrophic nutrition.

glands and vessels a specific staining for identification of the components of the fluid (Glycogen, Glycodelin A, Uteroferrin, etc.) beneath the implantation site (Fig. 1) would be of great interest. However, due to ethical constraints there is only very limited access to such specimens.

The question arises whether those glands that show destruction of their epithelium are still able to produce histiotrophic material to nourish the embryo. So far we observed destruction of the glandular epithelium only in the upper decidual aspects of the glands (functional endometrium/decidua), while the glandular aspects in the lower part of the decidua (basal endometrium/decidua) do not seem to be altered. Hence, histiotrophic material could be derived from the basal parts of the glands. Also, during early pregnancy the placenta is expanding quite fast and hence new glands are eroded frequently. This leads to the access of new histiotrophic material continuously during the first trimester. Finally, also the endoglandular trophoblasts may play a role in this nutritional scenario since extravillous trophoblasts are known to contain high amounts of glycogen (Boyd, 1959; Genbacev *et al.*, 1993; Babawale *et al.*, 2002).

Spencer (2014) stated that the blastocyst attaches to the luminal epithelium and then implants into the superficial functional layer of the decidua between the openings of the uterine glands. The implanted conceptus begins to grow and the syncytiotrophoblast invades into the superficial capillaries as well as the uterine glands, releasing the contents of both into the lacunae that will form the intervillous space (Spencer, 2014). Based on our current observations we want to extend this general concept and suggest that directly after implantation single trophoblasts (endoglandular trophoblasts) penetrate and invade uterine glands, after the very early penetration by the syncytiotrophoblast, thus enabling connection of the uterine glands toward the intervillous space (Figs 1 and 2). During the first trimester, single endoglandular trophoblasts first reach, then penetrate and open the uterine glands toward the intervillous space (Figs 3 and 4). Thereby, the early intervillous space is filled with secretions of the uterine glands (Burton *et al.*, 2002). In parallel, endovascular trophoblasts invade, line and subsequently plug uterine arteries prior to onset of the maternal circulation at the end of the first trimester (Hustin and Schaaps, 1987; Jaffe *et al.*, 1997; Kliman, 2000). Uterine glands are opened toward the intervillous space right from the beginning, spiral arteries not before the end of the first trimester. This is confirmed by our quantification of epithelial/endothelial cells replaced by EVTs, significantly more glandular than

vascular cross sections show partly replacement by EVTs during the first trimester of pregnancy (Fig. 4e). With our confrontation co-cultures of first trimester placental decidua with chorionic villi, we could show that endoglandular trophoblasts also remodel uterine glands *in vitro* (Fig. 5e). The confrontation co-cultures are harvested after 72 h of culture, at this time we were able to observe glands that were already replaced to one third. Hence, the process of complete replacement seems to run very rapidly.

Our data show that EVTs invade more structures in the maternal decidua than was known until recently, rather than specifically focusing on uterine spiral arteries. Taking together our results we suggest that EVTs originate from trophoblastic cell columns and migrate through the decidual interstitium, here being referred to as interstitial trophoblast (GA 5–12 weeks). From here either they may migrate toward uterine spiral arteries, where they replace vascular endothelial cells, and line and plug spiral arteries before the end of the first trimester (being referred to as endovascular trophoblast); only blood plasma may seep through the trophoblastic plugs toward the intervillous space prior to the establishment of the maternal blood flow (Huppertz *et al.*, 2014); or they migrate toward uterine glands, replace the glandular epithelium and thereby open the uterine glands directly to the intervillous space (being referred to as endoglandular trophoblast). Additionally, we suggest the following concept: uterine glands may be partly replaced and opened toward an already invaded uterine artery, resulting in a mixture of glandular secretion products and blood plasma, finally reaching the intervillous space (Fig. 6a and b).

In areas of trophoblast invasion, accumulated detached glandular epithelial cells were repeatedly observed in the glandular lumen (Figs 3–6), whereas in the non-invaded areas of the same uterus the glandular epithelium appeared still compact and well-proportioned. Also Jones *et al.* (2015) mentioned 'cells shed into the lumen', thus describing the disintegrated glandular epithelium (Jones *et al.*, 2015). Paracrine factors, secreted by endoglandular trophoblasts may lead to this disintegration of glandular epithelium in areas of invasion.

We observed that trophoblasts surrounding invaded glands also express MMP 9 (Fig. 6f). EVTs in general secrete various proteolytic compounds like matrix metalloproteases (MMPs), or members of the disintegrin and metalloproteases (ADAMs). On the one hand these proteases may play a crucial role in facilitating the migration through the decidual interstitium (Pollheimer *et al.*, 2014). On the other hand, EVTs may directly target uterine glands with these proteases, leading to a subsequent disintegration of glands, preparing them for the release of their secretion products toward the intervillous space. Hence, MMPs 2 and 9 are viewed as essential proteases for basal membrane-invasive events (Bischof *et al.*, 2000; Bischof and Irminger-Finger, 2005).

Moreover, we suggest that endoglandular trophoblasts secrete paracrine factors. These factors affect the epithelium of the uterine glands in the surrounding tissue; the glandular cells start to dissolve from the basement membrane. This may also be responsible for a reduction of the polarity of the glandular cells, thereby facilitating the invasion of the uterine glands. Recently, Buck *et al.* (2015) have presented a new confrontation co-culture model, spheroids of endometrial epithelial cells (EEC) are co-cultured together with the choriocarcinoma cell line AC-1M88. They demonstrated that maternal epithelial junction distribution and polarity affects the degree of trophoblast invasiveness. Less differentiated and polarized EEC facilitate the invasion of trophoblast cells, while strongly polarized spheroids are not invaded. Upon their results they suggest that endometrial glands prepare for trophoblast invasion by changes in the glandular EEC polarity (Buck *et al.*, 2015). Reduction in cell polarity is also a major prerequisite in cancer events. Epithelial cell polarity represents a barrier to the later stages of tumor development. Loss of epithelial cell polarity may have an important role both in the initiation of tumorigenesis and in later stages of tumor development, favoring the progression of tumors from benign to malignant (Royer and Lu, 2011). This strengthens the assumption, that invasion of uterine glands is a directed and important physiological process during early pregnancy.

In conclusion EVTs invade more structures in the maternal decidua than previously known. All potential routes of EVTs invasion known today are schematically represented in Fig. 7. Rather than specifically focusing on uterine spiral arteries, these cells also invade into uterine glands. Here we provide evidence that endoglandular trophoblasts are responsible for the opening of uterine glands toward the intervillous space. Paracrine factors may be responsible for the disintegration of uterine glands in the maternal decidua of the placental bed.

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Authors' roles

G.M.: conception and design, collection, analysis and interpretation of data, manuscript writing, revision of the manuscript. M.G.: conception and design, revision of the manuscript. M.S.: conception and design, collection and analysis of data. G.W.: conception and design, analysis of data, revision of the manuscript. B.H.: conception and design, data interpretation, revision of the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

None declared.

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