

The trophoblast plug during early pregnancy: a deeper insight

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Abstract During the first trimester of pregnancy, foetal endovascular trophoblasts invade into maternal spiral arteries, accumulate and form plugs in the lumen of the vessels. These plugs only allow blood plasma to seep through. Hence, during the first trimester of pregnancy, a first flow of fluids through the placental intervillous space is established, resulting in a physiological oxygen gradient between mother and foetus. The trophoblast plugs block spiral arteries until the beginning of the second trimester (11–14 weeks). In parallel, uterine glands are invaded and opened by endoglandular trophoblasts towards the intervillous space of the placenta, without showing the formation of plugs (Moser et al. in *Hum Reprod* 25:1127–1136, 2010, *Hum Reprod Oxf Engl* 30:2747–2757, 2015). This enables histiotrophic nutrition of the embryo prior to onset of maternal blood flow into the placenta. Failure of these endovascular and endoglandular invasion processes may lead to miscarriage or pregnancy disorders such as intrauterine growth restriction (IUGR). After dissolution of the plugs, the onset of maternal blood flow allows maternal blood cells to enter the intervillous space and oxygen concentrations rise up. In this study, we demonstrate for the first time serial cross sections through a trophoblast plug in a first trimester placental bed specimen. Invaded and plugged arteries as well as invaded uterine glands in week

11 of gestation are visualized with specific immunohistochemical double staining techniques. We show that spiral artery plugs appear throughout the placental invasion zone and illustrate erythrocytes stowed due to trophoblast plugs. In addition, we give evidence of the presence of MMP-1 in plugs of invaded spiral arteries. The results reveal a better understanding and a closer insight into the morphological appearance of trophoblast plugs and the consequences for placental and uterine blood flow.

Keywords Placenta · Spiral artery · Trophoblast plug · Endovascular trophoblast · Endoglandular trophoblast · Uterine gland

Introduction

Trophoblast invasion is a crucial event in human pregnancy and especially during the first trimester of pregnancy. The trophoblast subpopulations, villous and extravillous trophoblast, originate from cytotrophoblast stem cells (Bischof and Irminger-Finger 2005). In normal, uncomplicated pregnancies extravillous trophoblasts (EVTs) invade through the uterine interstitium towards decidua and myometrium (*interstitial trophoblast*). The invasion process is linked to appropriate cell to cell contact of EVT with the surrounding tissues, conducted by numerous cytokines like IL6 and uPAR expressed at the foetal–maternal interface (Weiss et al. 2016).

Prior to trophoblast invasion, the walls of spiral arteries undergo a variety of reorganization processes including widening of the vessel lumen (Brettner 1964), swelling of smooth muscle cells (Craven et al. 1998) and vacuolation of endothelial cells (Boyd and Hamilton 1967). During the invasion period, a subpopulation of EVT, *endovascular*

A dedication to Detlev Drenckhahn's integrated view on combining histochemistry, cell biology and molecular biology.

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Table 1 Antibodies used in immunohistochemistry

Antigen/antibody	Company	Dilution	Host/isotype
Cytokeratin 7 (KRT7)	Acris (Herford, Germany)	1:1000	Rabbit IgG pc
Major histocompatibility complex, class I, G (HLA-G)	BD Biosciences (Vienna, Austria)	1:1000	Mouse IgG mc
Matrix metalloproteinase 1 (MMP-1)	Protein Tech (Rosemont, USA)	1:500	Rabbit IgG pc
Von Willebrand factor (VWF)	Sigma Aldrich (St. Louis, USA)	1:1000	Rabbit IgG mc
Mouse IgG1 (DAK-GO1)	Dako (Carpinteria, USA)	1:100	Mouse IgG mc
Rabbit immunoglobulin fraction (X 0903)	Dako (Carpinteria, USA)	1:300	Rabbit IgG mc

trophoblasts, invades from the uterine interstitium into maternal spiral arteries and subsequently lines and remodels them. They penetrate through the endothelium and reach the lumen of the vessel where they accumulate and form trophoblast plugs (Huppertz et al. 2014; Kaufmann et al. 2003). These trophoblast plugs block the blood flow from the mother towards the placenta during the first trimester and finally disintegrate at the end of the first trimester, allowing maternal blood flow towards the intervillous space of the placenta (Jauniaux et al. 2000).

Besides invasion into spiral arteries, trophoblasts also invade towards uterine glands (*endoglandular trophoblast*). They attach, erode and replace the glandular epithelium, thus opening the uterine glands towards the intervillous space and thereby ensuring histiotrophic nutrition of the embryo prior to the onset of maternal blood flow (Moser et al. 2010, 2015).

In this study, we demonstrate the presence of trophoblast plugs and their progression within the placental bed tissues in immunohistochemically stained serial cross sections for the first time. We show that spiral artery plugs appear throughout the placental invasion zone and illustrate erythrocytes stowed due to trophoblast plugs. Furthermore we were able to detect MMP-1 in trophoblast plugs and thereby confirming the invasive phenotype of the endovascular trophoblasts.

Materials and methods

Tissue collection and processing

First trimester placental tissue (week 7–12 of gestation, $n = 40$) was obtained from elective termination of pregnancy. Informed consent was obtained with approval of the ethical committee of the Medical University of Graz. The tissue was rinsed in Hank's buffered salt solution (HBSS, Gibco Life Technologies, Austria), supplemented with 1 % penicillin/streptomycin and 1 % amphotericin B (Gibco Life Technologies). Tissue was subsequently collected for fixation in 4 % formalin for 24 h and embedded in paraffin.

After embedding, serial 5 μm sections were cut and placed on Superfrost Plus slides (Menzel, Braunschweig, Germany). For subsequent staining, 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. Primary antibodies were diluted in antibody diluent (Dako, Vienna). Table 1 lists details of all antibodies and the appropriate immunoglobulin G (IgG) negative control antibodies used in their respective dilutions. Sections were counterstained with Mayer's haemalaun and mounted with Kaiser's glycerol gelatine (Merck, Vienna Austria).

Immunohistochemical double staining

Immunohistochemical double labelling 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. Primary antibodies were diluted in antibody diluent (Dako). Binding of primary antibodies was visualized with the chromogens of the MultiVision kit, termed "LVBlue" and "LVRed".

One specimen (week 11 of gestation) showed outstanding plug structures. This specimen was examined in detail.

Microscopic evaluation

A microscope (model DM6000B; Leica) equipped with a motorized stage and a digital camera (model DP72; Olympus Austria GmbH, Vienna, Austria) were used for image acquisition. Images from serial sections were taken in

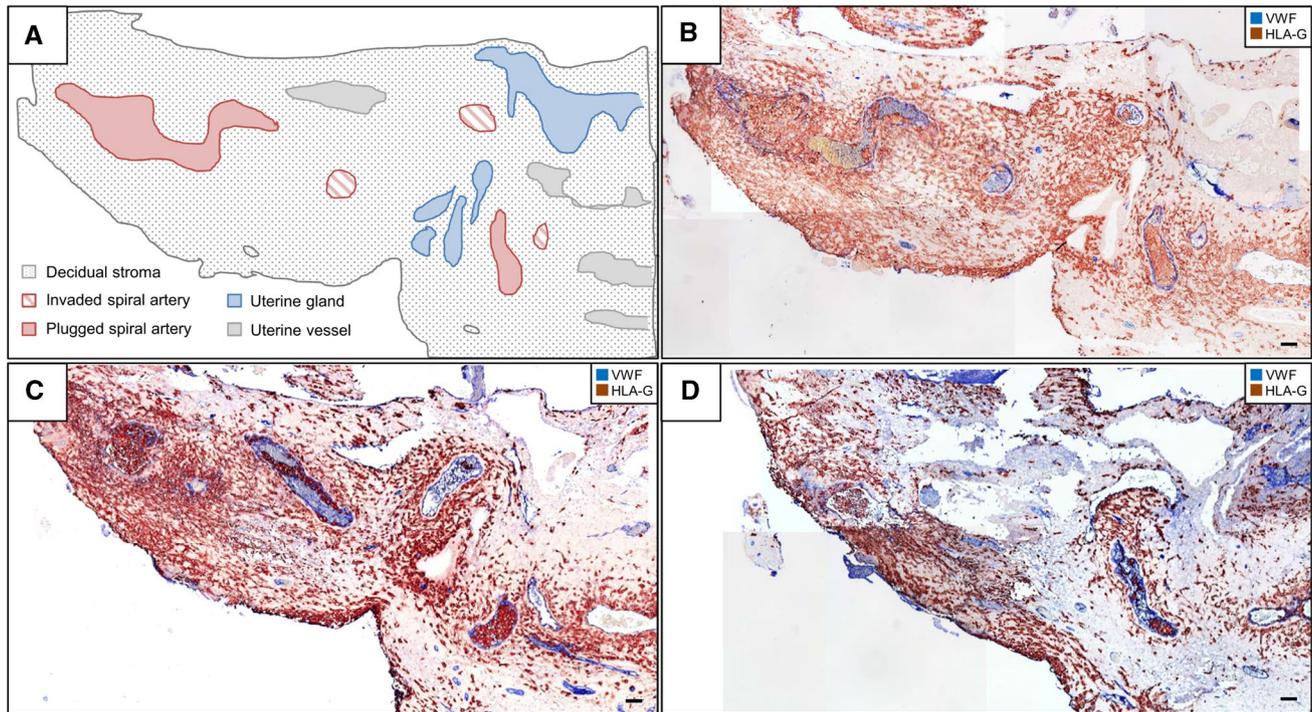


Fig. 1 Trophoblast invasion and arterial trophoblast plugs—overview. Immunohistochemical double staining of serial cross sections of invaded decidua basalis (gestational age 11 weeks) for major histocompatibility complex, class I, G (HLA-G) (brown, serves as marker for extravillous trophoblasts) and von Willebrand factor (vWF) (blue, serves as marker for vascular endothelial cells). No nuclear counterstain. **a** Schematic outline of structures of interest within the cross section in (**b**). Decidual stroma is *dotted*, plugged arteries are marked *red*, invaded arteries are marked with *red stripes*. Uterine glands are

marked in *blue* and uterine vessels in *grey*. **b** Corresponding histological cross section to the scheme in image **a**. Various invaded and plugged sectors of spiral arteries can be identified within the section. **c** Progression of the section in (**b**) with a distance of 55 μm. Alterations of plugs and invaded vessels and glands (specifically identified in respective stainings of serial sections, not shown) are present. **d** Further changes of invaded structure with a distance of another 60 μm. Scale bar represents 50 μm

different magnifications (50×–200×) and were manually stitched together with Microsoft Power Point 201 (Images in Figs. 1b–d, 2d, e).

One trophoblastic plug was followed in 35 consecutive sections until the plug was no longer visible. In 9 out of these slides, single trophoblasts were counted manually with an average of 58 cells per plug cross section. According to this number, we extrapolated a minimum number of extravillous trophoblasts within the plug.

Results

Invasion and plugging of uterine spiral arteries in the first trimester of pregnancy

We examined serial sections of decidua basalis containing trophoblast plugs. Due to the number of serial sections (5 μm in length) of the specimen, we examined a total of 120 μm of the respective plug area.

In the progression of the sections, extravillous trophoblast invasion and trophoblast plug(s) could be tracked and followed through the tissue.

For a better understanding and overview, Fig. 1a highlights the most prominent structures in the present placental bed section including invaded and plugged spiral arteries as well as uterine glands and vessels. Figure 1b shows the cross section reproduced in Fig. 1a. Immunohistochemical double staining of vWF and HLA-G shows two trophoblast plugs and invaded spiral artery structures within the placental tissue in this section. vWF also binds to fibrinoid structures, which is the reason for the blue staining inside all blood vessels. Furthermore uterine gland structures are partly invaded by extravillous trophoblast cells. Isotype-negative control antibodies did not reveal any staining (data not shown).

Figure 1c shows the consecutive region in another section in a distance of 55 μm. Parts of the big plug on the left side of the image can still be seen, and another segment of the big plug rises in the middle of the picture, where also uterine glands are strongly invaded by trophoblast cells.

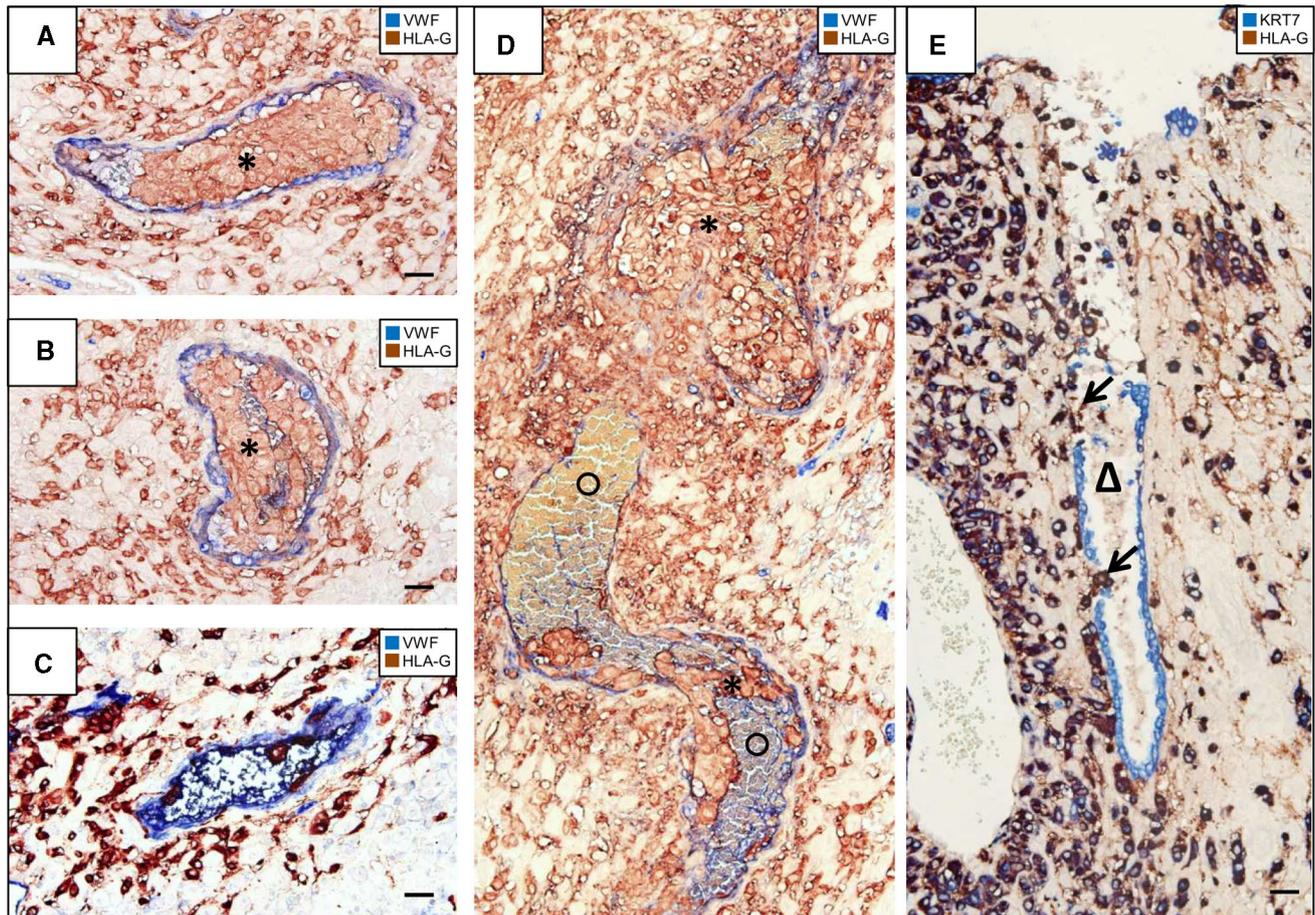


Fig. 2 Arterial trophoblast plugs and invasion of uterine glands—details. Immunohistochemical double staining of serial cross sections of invaded decidua basalis (gestational age 11 weeks) for major histocompatibility complex, class I, G (HLA-G) (brown, serves as marker for extravillous trophoblasts) and von Willebrand factor (vWF) (blue, serves as marker for vascular endothelial cells) (a–d) or cytokeratin 7 (KRT7) (blue, serves as marker for glandular epithelium here) (e). No nuclear counterstain. (a–c) Progression of a trophoblast plug (asterisk) in consecutive cross sections: In (a) and (b) the trophoblast plug

fills the complete lumen of the spiral artery, whereas in (c) there are only few endovascular trophoblasts left. (b) is in 55 μm distance to (a) and in 60 μm distance to (c). (d) highlights the most prominent plugged artery in the current placental specimen. Erythrocytes (circle) appearing yellow-greenish-blue are stowed by the trophoblast plug (asterisk). (e) demonstrates invasion of endoglandular trophoblasts (arrows) into a uterine gland (triangle). The gland is opened towards the intervillous space. Scale bar 50 μm

The trophoblast plug on the right side of the image shows another cut of the plug, smaller in diameter but still tightly packed with trophoblasts (Fig. 1c).

Another 60 μm further, we can only presume the presence of the big plug, while the one on the right side shows elongation in diameter, indicating another segment of a spiral artery, partly filled with trophoblasts.

In Fig. 2a–c, we demonstrate the progress of one plug on the right side of the sections in Fig. 1 in more detail. Figure 2a shows the plug tightly filled with trophoblasts. In a distance of 55 μm , the plug diameter changes and some erythrocytes in the lumen of the plug can be visualized (Fig. 2b). In Fig. 2c, we see the plug another 60 μm apart, showing just a few trophoblasts left in the

lumen of the vessel and more erythrocytes indicating the start of trophoblast infiltration upstream of the respective vessel.

Figure 2d shows a higher magnification of the big plug on the left side in Fig. 1. The longitudinal section of the spiral artery reveals the presence of the trophoblast plug. Erythrocytes (appear yellow-greenish) are stowed by the plug, and further trophoblasts are invading the vessel.

Besides spiral arteries also uterine glands are invaded by extravillous trophoblast cells (endoglandular trophoblast). Figure 2e shows a uterine gland next to a uterine vessel. The glandular epithelial cells are partly dissolved and replaced by trophoblast cells, opening the gland towards the intervillous space.

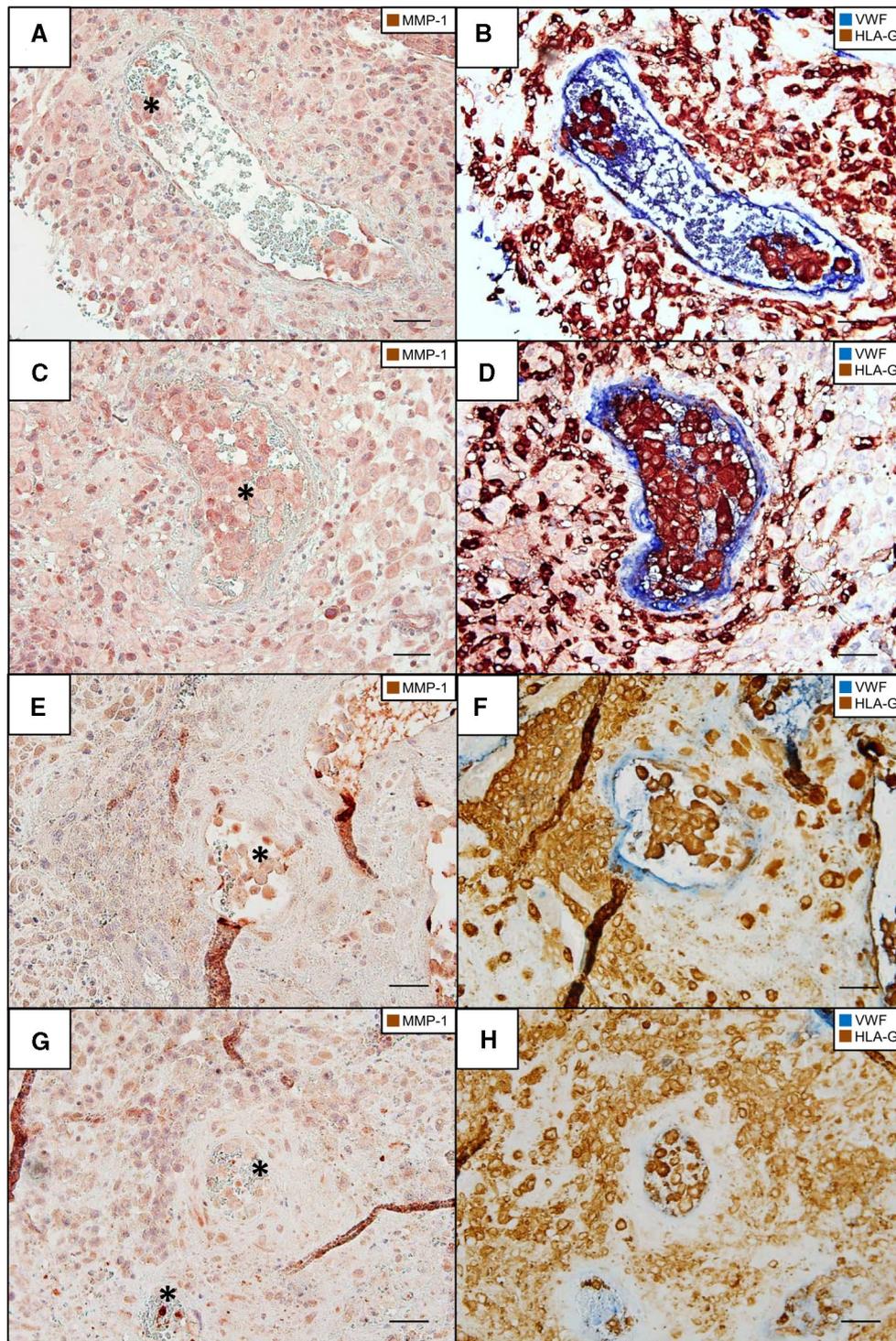


Fig. 3 MMP-1 expression in arterial trophoblast plugs. Immunohistochemical single (a, c, e, g) and double (b, d, f, h) staining of serial sections (a–b, c–d, e–f, g–h) of invaded decidua basalis (gestational age 11 weeks a–d and gestational week 7 e–h) for matrix metalloproteinase 1 (MMP-1) and for major histocompatibility complex, class I, G (HLA-G) (brown, serves as marker for extravillous trophoblasts) together with von Willebrand factor (vWF) (blue, serves as marker for vascular endothelial cells). Nuclei are counterstained with

Haemalum (a, c, e, g) or no nuclear counterstain (b, d, f, h). a, c, e, g Endovascular trophoblasts within trophoblast plugs (asterisk) are clearly positive for MMP-1. Additionally interstitial trophoblasts as well as decidual stroma cells are positive for MMP-1. b, d, f, h Immunohistochemical double staining in serial sections enables a clear classification of the assessed structures and cells and clearly confirms the extravillous origin of the trophoblasts composing the trophoblast plug. Scale bar 50 µm

MMP-1 presence in trophoblast plugs

To elucidate the presence of MMP-1 in extravillous trophoblasts in plugged spiral arteries, we performed immunohistochemistry in serial sections of the trophoblast plug. Figure 3a, c demonstrates the presence of MMP-1 in trophoblast plugs of spiral arteries and invading trophoblasts, respectively (week 11 of gestation). Additionally, serial sections were immunohistochemical double stained with antibodies against vWF and HLAG (Fig. 3b, d, f, h). The plugs show a clear expression of MMP-1, potentially higher than invading trophoblasts in the surrounding tissue. Also, specimen in week 7 of gestation show clear MMP-1 staining in trophoblast plugs (Fig. 3e, g). Isotype-negative control antibodies did not reveal any staining (data not shown).

Counting of trophoblast cells in a plug structure

We counted the trophoblast cells present in the plugs from the serial sections and roughly estimated that this trophoblast plug is composed of at least 720 single endovascular trophoblasts and extends over at least 115 μm .

Discussion

In the present study, we were able to demonstrate that trophoblast plugs block spiral arteries at the end of the first trimester (week 11 of gestation) by assessing serial sections of placental bed specimens with specific immunohistochemical single and double staining. In parallel, uterine glands were invaded and opened towards the intervillous space of the placenta by endoglandular trophoblasts, without showing the formation of plugs. Furthermore, the presence of MMP-1 in endovascular trophoblast within trophoblast plugs was verified.

During the first trimester, endovascular trophoblasts invade into the lumen of spiral arteries and form trophoblast plugs. These plugs only allow blood plasma to seep through to reach the intervillous space. Hence, a first flow of fluids through the placental intervillous space is established, resulting in a physiological oxygen gradient between mother and placenta and the foetus (Huppertz et al. *J Reprod Immunol* 2014). This oxygen gradient plays a central role in differentiation and invasion of extravillous trophoblasts. Growth and development of the embryo and the placenta are strongly influenced by the appropriate oxygen environment and changes of oxygenation throughout pregnancy (Huppertz et al. 2014). The physiological and therefore normoxic oxygen concentration for placenta and embryo until the end of the first trimester of pregnancy are below 20 mmHg barometric column (Jauniaux et al. 2000; Rodesch et al. 1992). Elevated oxygen levels during this period may cause

pregnancy complications and spontaneous abortion (Burton and Jauniaux 2004; Jauniaux et al. 2003).

Trophoblast plugs block the spiral arteries until the beginning of the second trimester (11–14 weeks) (Huppertz et al. 2014). For the first time, we show and visualize that erythrocytes (and putatively other blood cells) are stowed by the trophoblast plug, only allowing maternal blood plasma to seep through. This indicates the importance of the plugs to prevent the onset of placental blood flow prior to the end of the first trimester.

After dissolution of the plugs, the onset of maternal blood flow allows maternal blood cells to enter the intervillous space and oxygen concentrations rise up. The oxygen gradient disappears (Huppertz et al. 2009, 2014).

Starting from interstitial trophoblast invasion, EVT's do not only invade into uterine spiral arteries (*endovascular trophoblast*), another side branch of EVT invades into uterine glands (*endoglandular trophoblasts*), which are spread throughout the decidua during the first trimester of pregnancy (Moser et al. 2010). Until recently, it was commonly believed that the role of uterine glands is restricted to the process of implantation.

However, there is increasing evidence that they continue delivering nutritional components like proteins, carbohydrates and lipids and also immunosuppressive factors like MUC-1 into the intervillous space of the placenta post-implantation (Amoroso 1952; Brayman et al. 2004; Burton et al. 2007). The replacement of uterine glandular epithelial cells by endoglandular trophoblasts and the opening of uterine glands towards the intervillous space ensure histiotrophic nutrition of the embryo prior to onset of maternal blood flow during the first trimester (Moser et al. 2010). It has not yet been shown that failure in the replacement of uterine glands results in pregnancy pathologies, but it is tempting to speculate that early spontaneous miscarriages may also result from missing nutritional support following a failure of invasion of uterine glands.

In line with the findings of Moser et al. (2010), we were able to demonstrate invasion of extravillous trophoblasts into uterine glands and the aperture of these glands towards the intervillous space (Moser et al. 2015). We did not observe plug formation in glandular structures, which is not surprising due to the fact that histiotrophic nutrition of the embryo would be distorted by plugging the glands prior to the onset of maternal blood flow at the end of the first trimester.

It is commonly known that cytokines play essential roles in the process of trophoblast invasion. Numerous studies have shown the importance of cytokines released from trophoblasts most prominently cytokines like IL-8, IL-13 and RANTES (Naruse et al. 2010). Lately, also cytokines like GRO, IL6 and uPAR released from uterine arteries and veins have been linked to appropriate trophoblast invasion

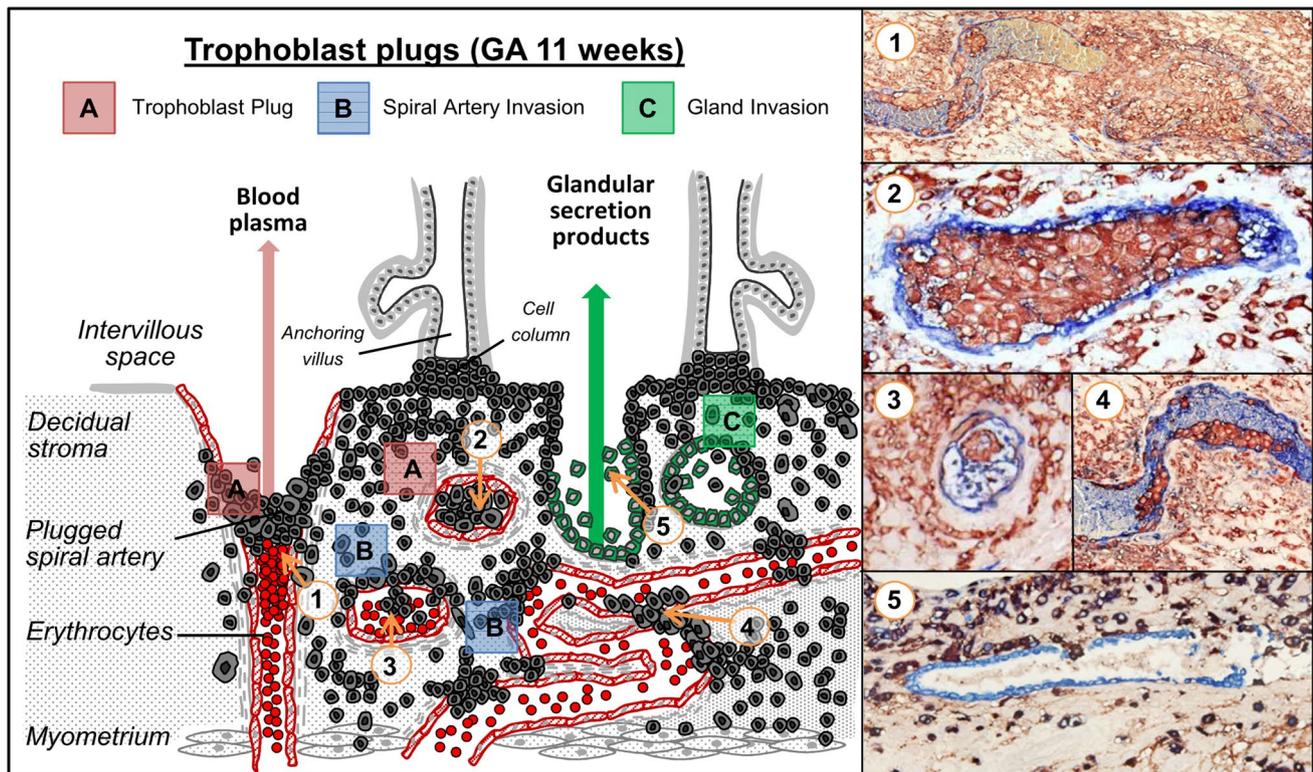


Fig. 4 Different perspectives of trophoblast plugs within spiral arteries. Trophoblast plugs (A) are composed of endovascular trophoblast cells. Depending on the stage of invasion and on the localization of sectioning the lumen of the spiral artery can be either completely filled with trophoblasts (1, 2) or partly infiltrated (3, 4). In a plugged

artery, the approaching erythrocytes are stowed by the trophoblast plug (1). Uterine glands are invaded and opened towards the intervillous space (C) by endoglandular trophoblasts. Glandular epithelial cells are replaced by endoglandular trophoblasts (5)

(Weiss et al. 2016). It is tempting to speculate that a strong interaction of cytokines released from trophoblasts on the one hand and cells of the invaded tissue on the other assures appropriate invasion of the tissue.

MMP-1, in particular, is known to be a prominent invasion marker. The proteinase degrades collagen (Hulbooy et al. 1997) and enables trophoblast cells to migrate through the uterine tissue. Deficient activity has been associated with the pregnancy pathologies like pre-eclampsia and intrauterine growth restriction (IUGR).

MMP-1 is commonly expressed in cells of the maternal–foetal interface, including EVT (Huppertz et al. 1998), cytotrophoblasts, syncytiotrophoblast (Vettraiño et al. 1996) and decidual cells (Lockwood et al. 2014). It has been reported that decidual endothelial cells express a low amount of MMP-1 in pre-eclamptic decidual tissue (Gallery et al. 1999). So far, the presence of invasion markers like MMP-1 in trophoblasts of endovascular plugs has only been proved in the rhesus monkey (Blankenship and Enders 1997). Here, we demonstrate the presence of MMP-1 also in human trophoblast plugs, indicating the invasive potential of endovascular trophoblasts and supporting the thesis that endovascular trophoblasts originate from interstitial

trophoblasts (concept of intravasation) (Kaufmann et al. 2003).

The scheme in Fig. 4 demonstrates the routes of trophoblast invasion and summarizes the different perspectives of trophoblast plugs with the corresponding in situ images (Fig. 4). In conclusion, the results reveal a better understanding and a closer insight into the morphological details of trophoblast plugs and the consequences for placental and uterine blood flow. In future studies, it will be interesting to gain more knowledge of characteristics of endovascular trophoblasts within the trophoblast plugs.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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