Dissertation

Alternative routes of trophoblast invasion: Investigations with novel confrontation co-culture model systems for early placentation

submitted by

Mag.rer.nat.

Gerit MOSER

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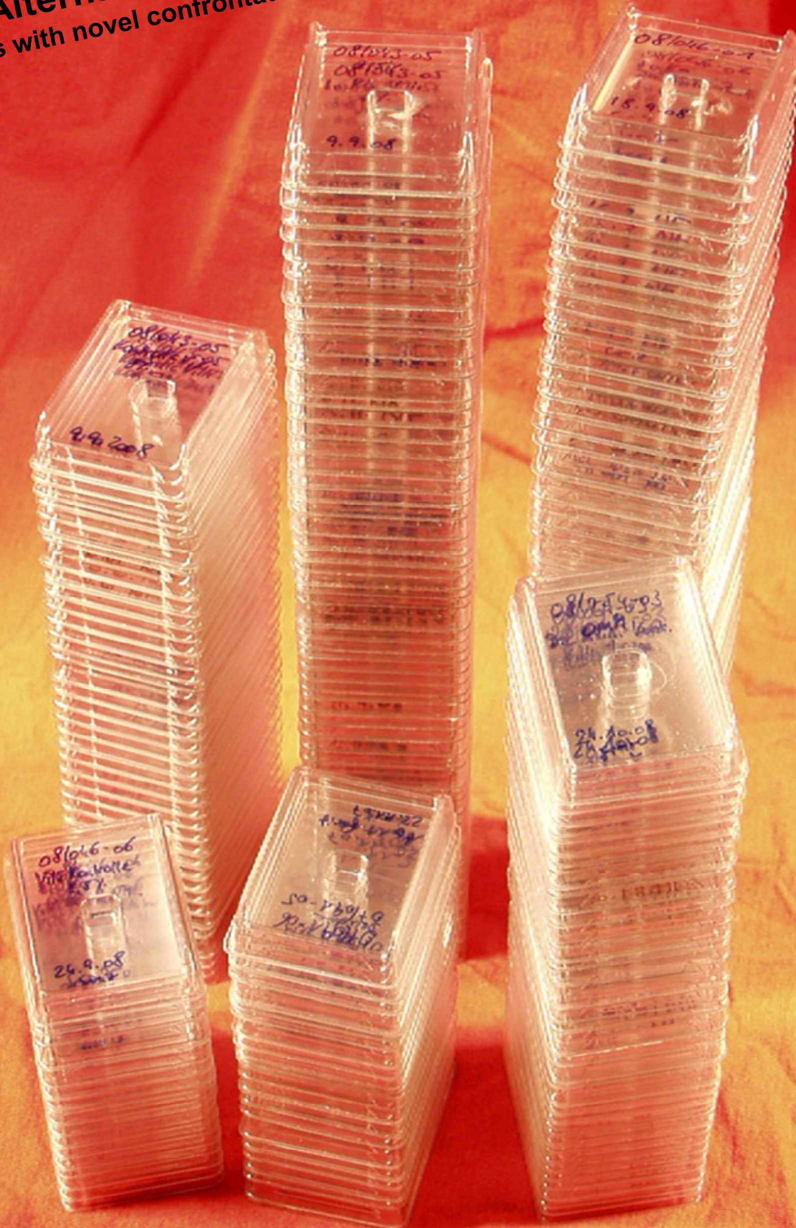
Alternative routes of trophoblast invasion:

Investigations with novel confrontation co-culture model systems for early placentation



Doctoral thesis

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Please note that parts of this thesis are already published in Moser G, Gauster M, Orendi K, Glasner A, Theuerkauf R, Huppertz B.: *Endoglandular trophoblast, an alternative route of trophoblast invasion? Analysis with novel confrontation co-culture models*. Human Reproduction, 2010 Feb 22, doi:10.1093/humrep/deq035

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1 Abstract

So far trophoblast invasion has been described to serve the attachment of the placenta to the uterus as well as transformation of the spiral arteries and later to establish the uteroplacental blood flow. These routes of trophoblast invasion seem to be clear, while specific invasive pathways still need further elucidation. Nutrition of the embryo during the first trimester is thought to be histiotrophic while the proof that extravillous trophoblasts invade uterine glands as well is still lacking. For elucidation of these processes novel *in vitro* confrontation co-culture models for mimicking early placentation were developed. With these models invasive behavior of extravillous trophoblasts into uterine tissues was monitored and attempts to influence invasion were made.

First trimester decidua parietalis and placental villous explants were directly confronted ("direct confrontation") and co-cultured for 72h, or confronted indirectly ("indirect confrontation") after 72h pre-culture for re-epithelialization of the decidua pieces. Confrontations were cryosectioned, stained by immunohistochemistry or immunofluorescent / immunohistochemical double labeling and compared with first trimester placentation sites *in situ*.

In both models (direct and indirect confrontation) confrontation of villous and decidual tissues resulted in formation of trophoblastic cell columns and their adhesion to decidual tissues. Extravillous trophoblasts invaded deeply into decidual tissues in direct confrontation assays, whereas they showed rather migration along the decidual epithelium during indirect confrontation. Sometimes they were situated between the decidual epithelial cells and the basement membrane of the latter cells. Routes of trophoblast invasion were followed and they were frequently detected in the decidual stroma in direct vicinity to glands, sometimes even replacing glandular epithelial cells. Similar observations were made in sections from the first trimester decidua / placental bed.

By using novel confrontation co-culture assays, a potential new route of extravillous trophoblast invasion was detected. It seems as if extravillous trophoblast cells break through the basement membrane of uterine glands to open their lumen towards the intervillous space. This supports the hypothesis of histiotrophic nutrition of the embryo prior to onset of maternal blood flow within the placenta.

2 Zusammenfassung

Die Invasion von Trophoblasten in maternale Gewebe während der frühen Schwangerschaft dient der Verankerung der Plazenta im Uterus sowie der Transformation der Spiralarterien und damit der nachfolgenden Etablierung der uteroplazentaren Durchblutung. Diese Routen der Trophoblastinvasion sind bereits gut beschrieben, andere Pfade bedürfen weiterer Erforschung. Der Embryo wird vor der Etablierung des uteroplazentaren Blutstromes mutmaßlich histiotroph ernährt Es gibt aber noch keinen Beweis für den Kontakt von Trophoblasten mit uterinen Drüsen. Um diese Prozesse aufzuklären, wurden neue Konfrontations-Kokulturmodellsysteme etabliert, um die frühe Plazentaentwicklung *in vitro* nachzustellen. Das Invasionsverhalten extravillöser Trophoblasten wurde untersucht, und in weiteren experimentellen Ansätzen auch beeinflusst.

Deziduastücke aus dem ersten Trimenon wurden entweder sofort (direkte Konfrontation) oder nach 72stündiger Vorkultur (indirekte Konfrontation) mit Zotten von derselben Plazenta kokultiviert. Während der Vorkultur reepithelialisierten die Deziduastücke. Die Konfrontationskulturen wurden gefriergeschnitten, mit verschiedenen immunohistochemischen bzw. immunofluoreszierenden Färbungen ausgewertet und die Ergebnisse mit *in situ* Proben aus dem ersten Trimenon verglichen.

Bei beiden Modellsystemen (direkte und indirekte Konfrontation) verwuchsen Dezidua und Zotten miteinander. Die Adhäsion der Gewebe resultierte immer in der Bildung von Zellsäulen mit Migration und/oder Invasion von mononukleären Trophoblasten. Bei der direkten Konfrontation war eine starke Invasion in das Deziduastroma zu sehen, wohingegen bei der indirekten Konfrontation eher Migration und weniger Invasion zu erkennen war. Einige extravillöse Trophoblasten waren auch zwischen Epithelzellen und der zugehörigen epithelialen Basallamina zu erkennen. Oft waren sie auch in der direkten Nähe von uterinen Drüsen zu finden, einzelne Trophoblastzellen ersetzten auch Drüsenepithelzellen.

Mithilfe der neu entwickelten Konfrontations-Kokulturmodellsysteme wurde eine potentielle neue Route der Trophoblastinvasion entdeckt. Es scheint, als ob extravillöse Trophoblasten durch die Basallamina von uterinen Drüsen brechen und damit deren Lumen in Richtung des intervillösen Raumes öffnen. Diese Ergebnisse unterstützen die Hypothese der histiotrophen Ernährung des Embryos vor Etablierung des uteroplazentaren Blutstromes.

3 Introduction

The development of the placenta begins once the blastocyst trophoblast establishes close and stable contact with the uterine luminal epithelium and mucosa, hence, as soon as the blastocyst implants. The blastocyst trophoblast makes up the outer wall of the blastocyst, which surrounds the blastocyst cavity and the inner cell mass. The trophoblast can be described as the precursor of the placenta. The inner cell mass, the so called embryoblast, is composed of a small group of larger cells. The embryo, umbilical cord and amnion are derived from these cells. In addition, both embryoblast-derived mesenchyme and embryoblast-derived blood vessels contribute to the formation of the placenta. Normally the blastocyst is orientated in a way that the part which contains the embryonic pole attaches to the endometrium (Benirschke *et al.*, 2006).

Implantation in the human begins six to seven days after fertilization and can be divided into three stages. Apposition comes first, which is the beginning of a still unstable adhesion of the blastocyst to the uterine wall. Apposition of the blastocyst is followed by stable adhesion, which is characterized by an increased physical contact between the blastocyst and the uterine epithelium. Now the embryonic pole is orientated towards the epithelium. During attachment and invasion of the endometrial epithelium, the trophoblast cells at the implanting embryonic pole of the blastocyst show increased proliferation resulting in a double-layered trophoblast (Benirschke *et al.*, 2006).

The differentiation of trophoblast into two fundamentally different subtypes – named syncytiotrophoblast and cytotrophoblast – begins. The third stage is the invasion process, where the multinucleated syncytiotrophoblast penetrates through the uterine epithelium (Vigano *et al.*, 2003). As soon as the early embryo has penetrated the epithelium and is surrounded by the endometrial connective tissues, the syncytiotrophoblast forms a complete layer surrounding the early embryo, this layer is encasing the conceptus completely. In the second row beyond the syncytiotrophoblast there are the mononucleated cytotrophoblasts, without any contact to maternal tissues. The expanding syncytiotrophoblast is continuously fed by dividing and fusing cytotrophoblasts.

Figure 1 gives a schematic overview of the human implantation process. During the last step (villous formation) the conceptus is surrounded by a complete mantle of syncytiotrophoblast, and mononucleated cytotrophoblasts do not come into contact with maternal tissues at this stage of development.

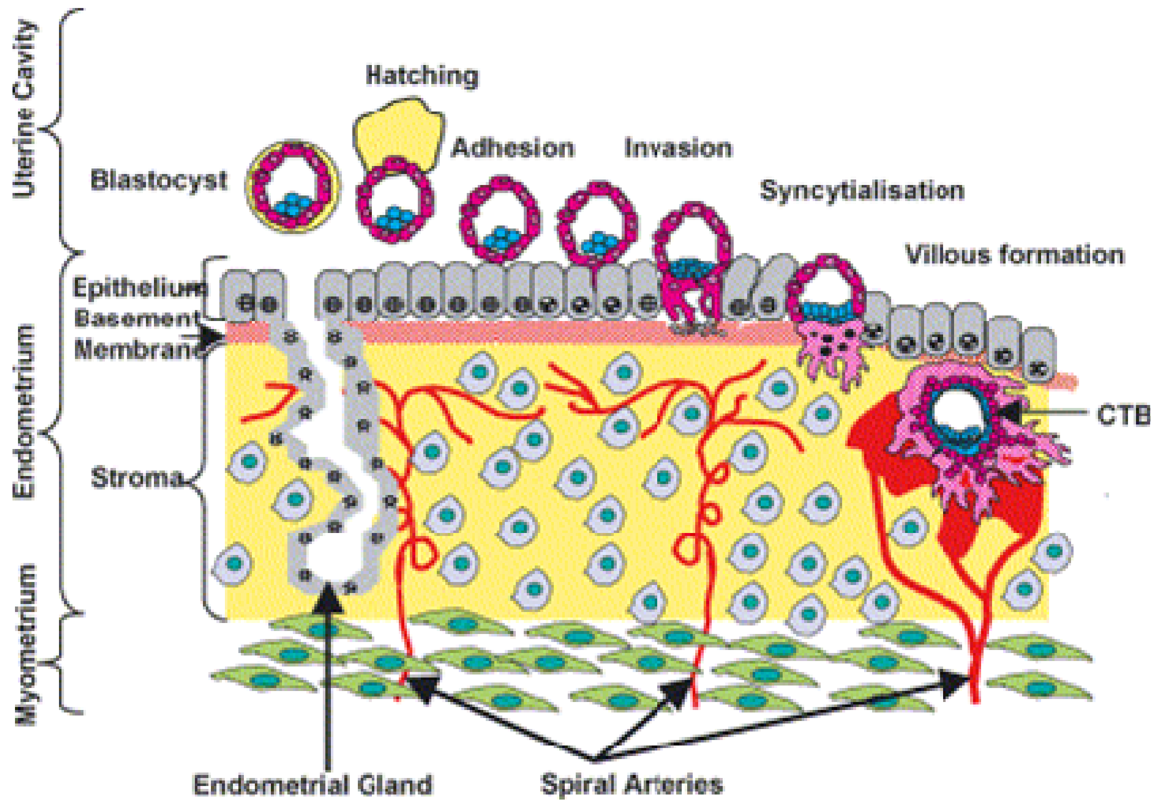

Figure 1: Human implantation. When the blastocyst reaches the uterine cavity, the embryonic pole of the blastocyst orientates towards the uterine epithelium and the blastocyst adheres to it. Prior to invasion the trophoblast differentiates into syncytiotrophoblast and cytotrophoblast. During early villous formation the conceptus is completely mantled by syncytiotrophoblast (Bischof and Irminger-Finger, 2005).

Eight days after conception lacunae start to develop out of small intrasyncytial vacuoles on the surface of the implanting blastocyst. This so called lacunar stage of placentation lasts from day 8 to day 13 post conception. The lacunae are separated from each other by syncytial trabeculae, which start to be invaded by cytotrophoblasts after day 12 post conception. At about day 15 post conception mononucleated cytotrophoblasts start to invade the decidua. Throughout the course of the first trimester they will invade the whole thickness of the endometrium, the inner third of the myometrium and the uterine vasculature at the site of the placental bed, i.e. the uterine tissues directly underneath the placental attachment site. This is a key event during early implantation and placentation and responsible for further invasion of the blastocyst, for adaptation of the maternal vessels to pregnancy conditions as well as for anchorage of the developing placenta. Too excessive or too shallow invasion is associated with pathologies of pregnancy.

At about day 13 post conception increased cytotrophoblastic proliferation with subsequent syncytial fusion takes place in the trabeculae. Longitudinal trabecular growth can be observed as well as formation of blindly ending side branches which protrude into the lacunae. These formations are invaded by cytotrophoblasts and as long

as they are pure trophoblastic structures, they are called primary villi. Out of these primary villi the branching of villous trees starts. At the same time the lacunar system transforms into the intervillous space. When early villous structures come into contact to the decidual tissues of the developing basal plate, they adhere to the maternal tissues and are called anchoring villi. From the tip of anchoring villi cytotrophoblasts proliferate and start to migrate outwards to trophoblastic cell columns. From these cell columns extravillous trophoblasts migrate into the decidua, invade the decidual interstitium and the maternal spiral arteries, being subsequently responsible for the establishment of the uteroplacental circulation. There are various subtypes of extravillous trophoblasts known, an overview and description of the subtypes is shown in figure 2. As soon as cytotrophoblasts turn into invasive, extravillous trophoblasts they lose their proliferative potential (Benirschke *et al.*, 2006; Huppertz, 2008; Norwitz *et al.*, 2001).

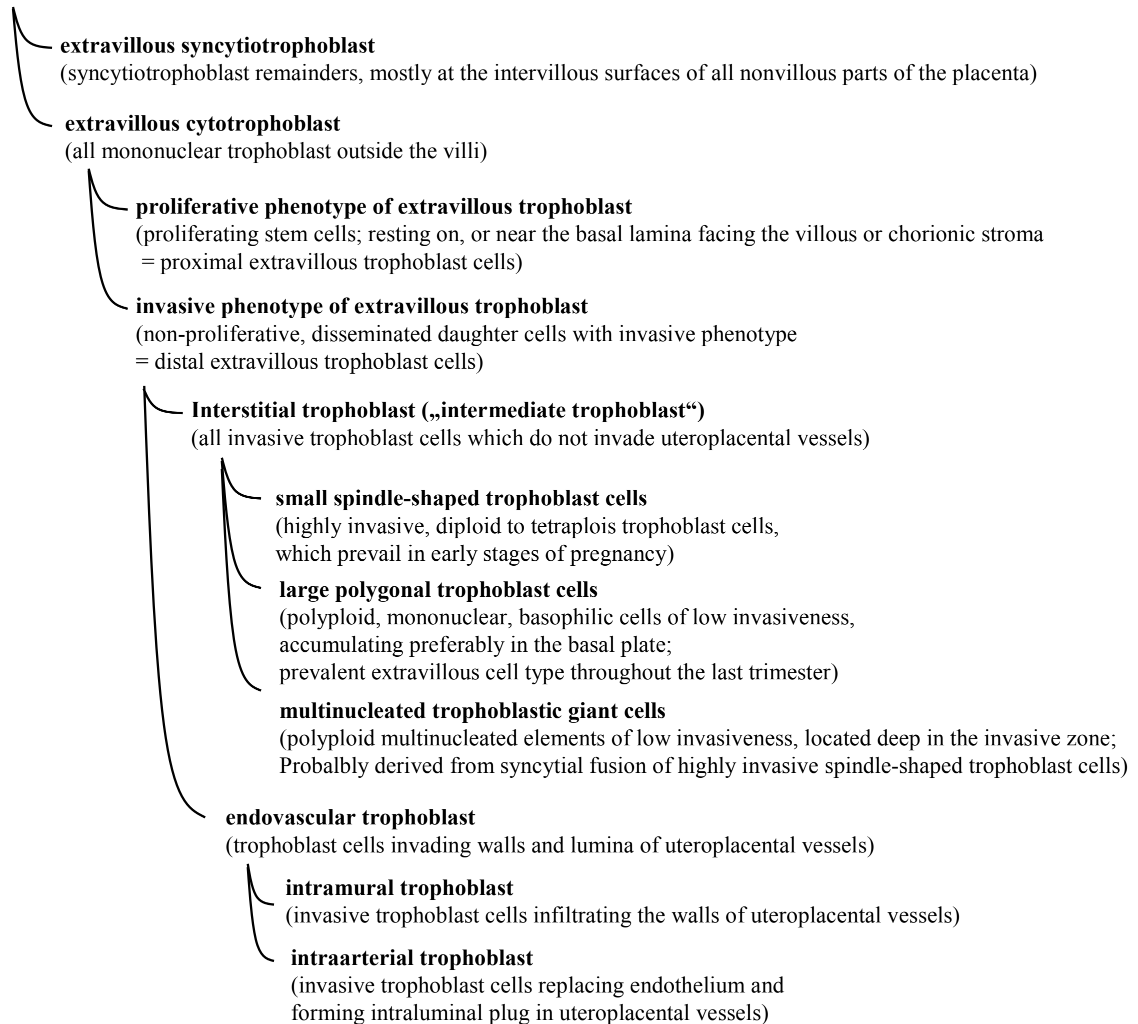

Figure 2: Nomenclature of various subtypes of trophoblast cells outside the villous trees (so-called extravillous trophoblasts) (modified from (Frank and Kaufmann, 2006))

At about two weeks after fertilization extravillous trophoblasts start to infiltrate maternal tissues by invading into 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 fetus (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 Leucemia inhibitory factor (LIF) and mucin-1 (MUC-1) (Brayman *et al.*, 2004; Carson *et al.*, 2000; Seppala *et al.*, 1998).

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. demonstrated that the clear fluid found in the intervillous space of a first trimester placenta contains secretory components of the uterine glands (Burton *et al.*, 2007). It is, however, unclear as to how these glandular products reach the intervillous space of the placenta. It may be speculated that the invasive extravillous trophoblast 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.

However, the process of human implantation and subsequent early placentation is still a kind of black box in research. Due to ethical reasons it is not possible to investigate the process *in vivo*. There are only very few specimens existing which document the first weeks of embryonic development in humans and the important step of the initial adhesion of the blastocyst to the uterine epithelium has never been observed (Norwitz *et al.*, 2001).

Animal models would be an alternative, but there is no ideal model for human placentation. Here I have listed some examples for strengths and weaknesses of animal models for human placentation;

(1) There are some analogies between mouse and human in placental cell types, but there is only limited trophoblast invasion in the mouse.

(2) Sheep basically serve as a model in fetal physiology. In the ovine placenta uterine vessels are not invaded and there is a differing immunology of pregnancy.

(3) In monkeys the important difference to humans is the absence of interstitial trophoblast cells.

(4) Aside, there are some alternative models to be mentioned, mainly for studies of comparative placentation and for understanding on how the placenta has evolved, like the tree shrew, the lesser hedgehog tenrec and the nine-banded armadillo (Carter, 2007).

Anyway, results obtained from animal models cannot be directly transferred to the human situation. Thus, other model systems for human early placentation are needed. To imitate human implantation and early placentation, co-culture model systems using decidual tissues as matrices for trophoblast invasion have been used. Such model systems have been successfully applied 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).

4 Aims

The aim of the work was to develop novel *in vitro* model systems for early placentation. Novel assays for confrontation co-culture of villi and decidua to monitor invasion of extravillous trophoblasts into decidual tissues were established and described. With these model systems questions concerning invasive behaviour of extravillous trophoblasts were addressed, like factors potentially influencing migratory and invasive properties as well as direction of invasion. It was attempted to influence invasiveness of trophoblasts for example by inducing oxidative stress in the model systems. Trophoblast invasion into the decidual interstitium and also towards uterine glands was monitored and visualized with different techniques. The data derived from the confrontation co-culture model systems may provide first evidence for the infiltration of the lining of the glandular epithelium by extravillous trophoblast, thus opening the way for secretion products to reach the intervillous space.

5 Methods

5.1 Tissue collection

First trimester placentas were obtained from elective terminations of pregnancies (gestational age (GA) 5 to 11 weeks, N = 36). Invaded decidua basalis was obtained from 10 placentas with gestational age of 6, 7, 8, 9 and 11 weeks. For the *in vitro* experiments only samples from placentas with a GA of 6 to 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 (HBSS), supplemented with 1% penicillin/streptomycin and 1% amphotericin B (PAA Laboratories, Austria), placed in culture media (DMEM high glucose with sodium pyruvate, without L-glutamine, supplemented with 10% FCS, 2% L-glutamine, 1% penicillin/streptomycin, 1% amphotericin B (PAA), 20ng/ml progesterone and 3ng/ml 17-β-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 HLA-G immunohistochemistry. From every placenta various tissue samples were collected for fixation in 4% paraformaldehyde and subsequent paraffin embedding. For the preparation paraffin specimens, the tissues were fixed in 4% neutral buffered formalin for at least 24h and routinely embedded using an automatic tissue processor (Shandon Citadel, Thermo Scientific, USA). Additional tissue samples were embedded in tissue freezing medium (TissueTek®; Sakura Finetek Inc, Torrance, USA) and stored at -70°C.

5.2 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 ~1mm in diameter. From the placenta proper villi were cut in pieces of corresponding size (15 to 20mg moist mass).

For direct confrontation (without decidual epithelium) one piece of decidua was co-cultured with one villous explant in 500µl culture media in a 2ml reaction tube

(Eppendorf, Germany). Before culture the reaction tubes were perforated for gas-exchange. For indirect confrontation (with decidual epithelium) decidua pieces were pre-cultured as previously described (Helige et al., 2008). Briefly, decidua pieces were transferred into 25ml spinner flasks and stirred at ~130rpm with a magnetic stirrer system (Telesystem 06.40 and Telemodul 40C, H+P Labortechnik AG, Oberschleißheim, Germany) for 72h at 37°C and 5% CO_2. Culture media was changed once after 24h. Villi were pre-cultured in parallel in 20ml culture media in 10ml petri dishes (Greiner Bio-one, Kremsmuenster, Austria) at 37°C and 5% CO_2 and 2.5% O_2 in an Xvivo incubation system (Model G300C, BioSpherix Ltd, Lacona, USA). After 72h pre-culture the decidua 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 72h at 37°C and 5% CO_2. Optionally, villi from one placenta were pre-cultured at 37°C with 5% CO_2 and 2.5% O_2 for 72h and then confronted with fresh decidua from another placenta. Control cultures with decidual and villous explants cultured alone were set up in parallel with each confrontation experiment. For a schematic overview of the experimental setup see figure 3.
After confrontation culture the adhered tissues were routinely embedded in tissue freezing medium (TissueTek), frozen at -80°C 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. For a detailed description please see protocols 13.1 and 13.2.

5.3 Immunohistochemistry / Immunofluorescence

5.3.1 Preparation of sections

Serial 5µm sections 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 was routinely assessed using anti HLA-G antibodies. For immunohistochemistry and immunofluorescence slides were thawed, air dried, fixed in acetone, air dried again for 5min 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 7min at 120°C before immunohistochemistry.

5.3.2 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 IgG fractions as isotype controls (Tab. 1). For a detailed description please see protocols 13.3 and 13.4.

5.3.3 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 a detailed description please see protocol 13.5. Invasion of extravillous trophoblasts into glands was quantified in immunohistochemically double labeled sections of invaded decidua basalis (10 cases, 6 to 11 weeks). Glandular cross sections associated with or invaded by extravillous trophoblasts were counted and compared with the total number of glandular cross sections in the invaded area of the tissue section.

5.3.4 Immunoflourescent double staining

For immunofluorescent double labeling the slides were incubated with an Ultra V-Block (Thermo Scientific) containing 10% human AB-serum for 8min at room temperature. Slides were rinsed in PBS three times before applying both primary antibodies for 30min at room temperature. Primary antibodies were diluted in antibody diluent (Dako). Slides were rinsed again in PBS three times and were then incubated for 30min at room temperature with 20µg/ml fluorescent labeled secondary antibodies (Alexa Fluor®555 goat anti-mouse IgG and Alexa Fluor®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 30min at room temperature, followed by incubation with 20µg/ml Alexa Fluor®555 goat anti-mouse IgG, followed

by incubation with a directly FITC conjugated anti-cytokeratin antibody. Slides were counterstained with 4,6 diamidino -2-phenylindoledihydrochloride (DAPI) (diluted 1:2000 in PBS; Invitrogen) for 10min at room temperature. Slides were rinsed in deionized water and mounted with ProLong® Gold antifade reagent (Invitrogen). For a detailed description please see protocols 13.6 and 13.7. Sections were assessed with an Axiophot microscope and photographs were taken using an AxioCam HRc digital camera (Zeiss, Oberkochen, Germany).

5.3.5 Immunofluorescent triple staining

For immunofluorescent triple staining, slides were incubated with Ultra V-Block (Thermo Scientific) containing 10% human AB-serum for 8min at room temperature. Slides were rinsed in PBS three times before applying two primary antibodies (MEM-G9 and entactin) for 30min at room temperature. Primary antibodies were diluted in antibody diluent (Dako). Slides were rinsed in PBS three times and were then incubated for 30min at room temperature with fluorescent labeled secondary antibodies (20µg/ml Alexa Fluor®555 goat anti-mouse IgG, Invitrogen, Lofer, Austria and 5µg/ml Cy 5 conjugated AffiniPure goat-anti-rabbit IgG, Jackson ImmunoResearch Laboratories Inc, West Grove, USA). Slides were washed in PBS and incubated with a directly FITC conjugated anti-cytokeratin antibody. Slides were rinsed in deionized water and mounted with ProLong® Gold antifade reagent (Invitrogen). For a detailed description please see protocol 13.8. Confocal laser scanning microscopy was performed on a Leitz/Leica TCS-SP2 microscope.

5.3.6 Immunofluorescent double staining – whole mount

For getting a three dimensional impression of confrontations immunofluorescent staining of whole tissue pieces was carried out as follows. Instead of cryosectioning whole indirectly confronted tissues were washed three times in Hanks´ BSS and fixed in ice-cold acetone for 30 min. Afterwards tissues were washed three times in TBS-T and then incubated overnight in the primary antibody solution (RPE-conjugated anti-human HLA Class I antigen and FITC-conjugated cytokeratin in antibody diluent) at 4°C on a shaking platform. Tissues were washed three times in TBS-T and counterstained with ToPro3 (Molecular Probes Cat.No. T-3605, Leiden, Netherlands) diluted 1:500 in 1xPBS for 8min. Tissues were washed again 3 times in TBS-T. Slides were prepared with reinforcing rings as spacer and tissues were mounted in the centre with ProLong®

Gold antifade reagent (Invitrogen) and assessed on a confocal laser scanning microscope (Leitz/Leica TCS-SP2). For a detailed description please see protocol 13.9.

Another approach for getting a three dimensional impression of the confronted tissues was to produce thick sections of the invasive front of confronted tissues as follows. Normal direct confrontations were cut in thick tissue sections on a cryostat (HM 560 V, Microm, Walldorf, Germany). Object temperature was set to -15°C and blade temperature was set to -10°C. Thickness was set to 100µm and sections were transferred with dissecting needles onto Superfrost plus slides. Tissue sections were air dried overnight at room temperature, then acetone fixed and stained or stored at -20°C. After storage slides were thawed for 5min, air dried for 10min, fixed in acetone for 5min, air dried for 5min and then washed in TBS-T three times for 5min. Primary antibody solution (mouse monoclonal MEM/G-9 in antibody solution, for dilution factor see table 1) was applied overnight at 4°C. Slides were rinsed in TBS-T three times and were then incubated for 30min at room temperature with a fluorescent labeled secondary antibody (20µg/ml Alexa Fluor®488 goat anti-mouse IgG, Invitrogen, Lofer, Austria). Tissues were then washed again three times in TBS-T and counterstained with ToPro3 (Molecular Probes Cat.No. T-3605, Leiden, Netherlands) diluted 1:500 in 1xTBS for 8min. Slides were rinsed in deionzed water and mounted with ProLong® Gold antifade reagent (Invitrogen). For a detailed description please see protocol 13.10. Confocal laser scanning microscopy was performed on a Leitz/Leica TCS-SP2 microscope.

5.4 De-epithelialization

Decidua pieces were dissected and precultured for re-epithelialization as described above. The uterine luminal epithelium was removed either by mechanical disruption or by loosening epithelial cells with trypsin. For mechanical disruption of epithelium re-epithelialized decidua pieces were pipetted ten times up and down through a narrow pipette tip. For trypsination the re-epithelialized decidua pieces were washed twice in Hanks' BSS (PAA laboratories, Pasching, Austria) to remove adherent FCS from the culture media and then incubated in 5ml 10x Tryspin-EDTA (0.5% Trypsin 0.2% EDTA, Cat.No L11-003, PAA laboratories, Austria) for 3min at 37°C in a humidified incubator. Concentration of trypsin was determined as used before for denudation of villi by Baczyk *et al.*, 2006. During trypsinization tissues were shaken gently once, control decidua pieces were incubated in 1x HBSS in parallel. After trypsinization decidua pieces were washed twice in fresh culture media to stop enzyme activity. De-

epithelialized decidua pieces were then confronted according to the protocol for indirect confrontation. Control confrontations were carried out with normal re-epithelialized decidua pieces. For a schematic overview of the experimental setup see figure 8. For a detailed description please see protocols 13.11 and 13.12.

5.5 Oxidative stress

Direct confrontations were stressed either before confrontation co-culture or during the whole duration of confrontation culture. For stressing before confrontation decidual and villous tissues were dissected and stressed separately for one hour in normal culture media (see above) supplemented with either 0, 2 or 4mM H_2O_2. Afterwards tissues were washed once in fresh media and then confronted according to the protocol for direct confrontation. Stressed confrontations were prepared from a total of three placentas. From each placenta 10 confrontations were set up at 0, 2 and 4mM H_2O_2, respectively. After confrontation culture tissues were cryosectioned and assessed immunohistochemically. In a different approach decidual and villous tissues were used for confrontation culture for 72h in culture media supplemented with either 0, 2 or 4mM H_2O_2. For a schematic overview of the experimental setup see figure 12. For a detailed description please see protocols 13.13 and 13.14. Cell viability was assessed by determination of LDH in culture supernatant using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Japan) according to the manufacturer’s manual. The LDH-release was adjusted to the weight of cultured tissue. Significance was tested with the student-T-test. P-Values of <0.05 were considered significant.

Table 1: Primary antibodies used in immunohistochemistry / immunofluorescence

Antibody Clone / Cat No	Company	Dilution IHC cryo	IF cryo	IHC ffpe	Host / Isotype
Cytokeratin 7 (OV-TL 12/30)	Thermo Scientific (Fremont, USA)	1:10000	--	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 Repulic)	1:2000	1:500	--	Mouse IgG mc
HLA-G (4H84)	Exbio (Prague, Czech Repulic)	--	1:5000	1:10000	Mouse IgG mc
Human Ki67 (MIB-1)	Dako (Carpinteria, USA)	1:100	--	--	Mouse IgG mc
FITC-conjugated Cytokeratin (MNF116)	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
Alexa Fluor® 555 goat anti-mouse IgG (SKU#A-21422)	Invitrogen (Lofer, Austria)		1:100		Goat
Alexa Fluor® 488 goat anti-rabbit IgG (SKU#A-11008)	Invitrogen (Lofer, Austria)		1:100		Goat
Cy™5-conjugated goat-anti-rabbit IgG (111-175-047)	Jackson ImmunoResearch Laboratories Inc (West Grove, USA)		1:300		Goat
RPE-conjugated Anti-Human HLA Class I Antigen (W6/32)	Dako (Carpinteria, USA)		1:10		Mouse IgG mc
Pan-reactive Cytokeratin Alexa Fluor® 488 conjugated (C-11)	Exbio (Prague, Czech Repulic)		1:10		Mouse IgG mc

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

6 Results

6.1 Confrontation co-culture model systems

Confrontation co-culture model systems for the investigation of extravillous trophoblast invasion into decidual tissues were developed. 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 top of a piece of decidua parietalis (without epithelium) and cultured for 72h. Alternatively, decidua pieces were pre-cultured under constant agitation for 72h for re-epithelialization and then confronted with villous explants from the same pregnancy (indirect confrontation) (Fig. 3).

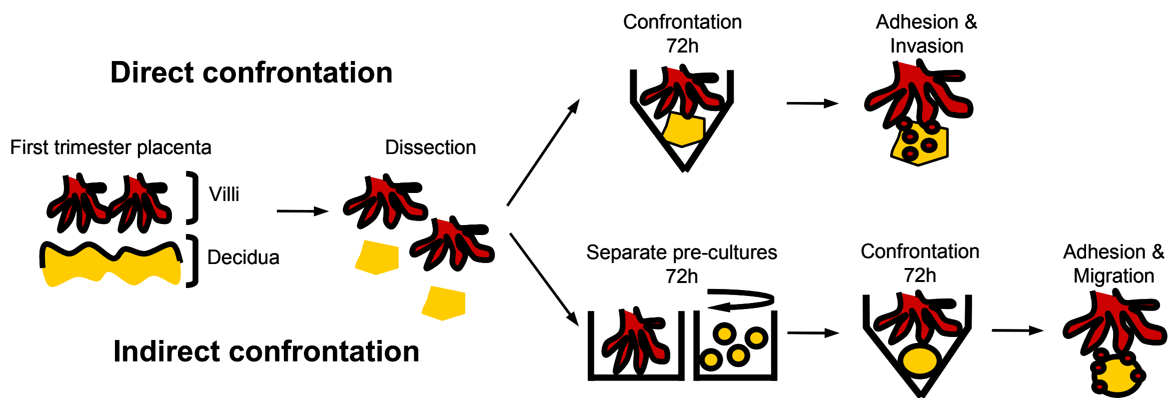

Figure 3: Schematic representation of the experimental procedures for direct and indirect confrontation co-cultures. First trimester decidua parietalis and placental villous explants were dissected and then directly confronted and co-cultured for 72h, or confronted indirectly after 72h pre-culture for re-epithelialization of the decidua pieces. (This scheme has been published in Human Reproduction (Moser *et al.*, 2010))

Re-epithelialization of cultured decidua within 2 to 3 days was already described earlier in similar model systems (Helige *et al.*, 2001; Vicovac *et al.*, 1995), and could be confirmed in our model systems: At the starting time of the culture there is no epithelium covering the decidua piece, only glandular cells stain positive for cytokeratin7 (Fig 4A). A cross section of a decidua piece cultured for 50h shows beginning outgrowth of cytokeratin 7 positive epithelial cells from a gland opening (Fig. 4B). After 72h of culture under constant agitation the decidua piece has completely re-epithelialized (Fig. 4C). The replenished epithelium after preculture 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. 4D) as well as of uncultivated decidua (Fig. 4E). In both cases cytokeratin 7 positive epithelial cells are situated on 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 top of it. Thus, it may well serve as a model for uterine epithelium.

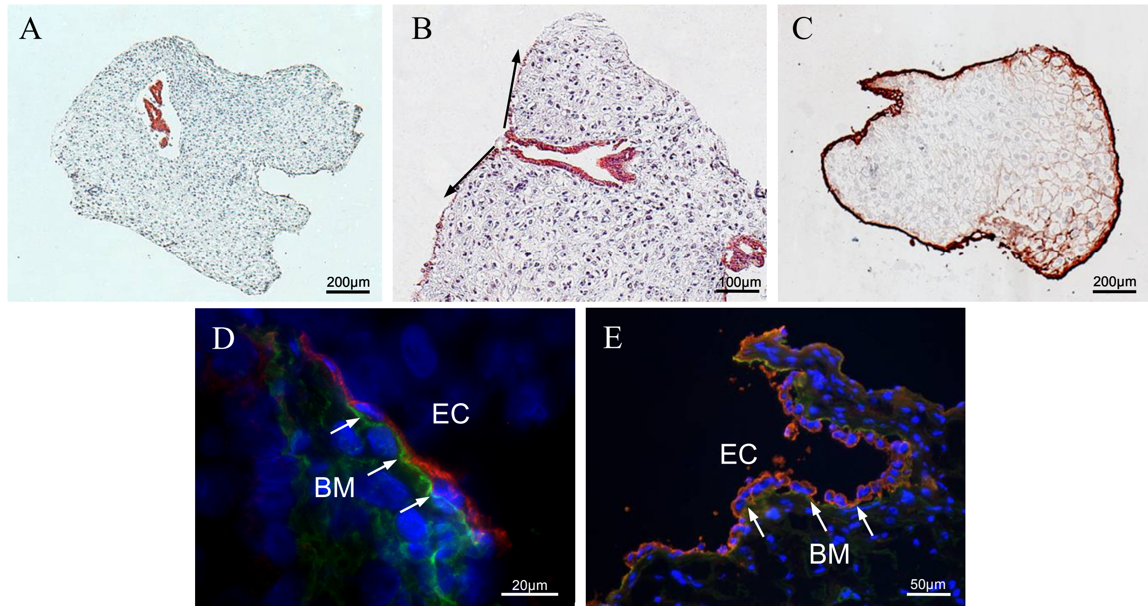

Figure 4: Re-epithelialization of decidua pieces (A, B, C) Immunohistochemical staining with antibodies against cytokeratin 7. (A) Freshly dissected piece of decidua parietalis, only glandular cells in the center of the piece are positive for cytokeratin 7. (B) Re-epithelialization of decidua (GA 8 weeks) after 50h of culture: Outgrowth of cytokeratin 7 (Ck 7) positive epithelial cells, arrows indicate putative growth direction of epithelial cells. (C) Completely re-epithelialized decidua after 72h of culture stained for cytokeratin 7. (D, E) Immunofluorescent double staining with antibodies against cytokeratin 7 to visualize epithelial cells (red) and entactin to visualize the basement membrane (green). The replenished epithelium (arrows) of cultured decidua (D) is similar to the epithelium of uncultivated decidua (E). Nuclei were counterstained with hemalaun (A-C) or DAPI (D, E). BM = basement membrane, EC = epithelial cells (part of this data has been published in Human Reproduction (Moser *et al.*, 2010))

In both model systems confrontation of villous explants and decidual pieces resulted in adhesion of tissues (Fig. 5A). The anti-HLA-G antibody MEM-G9 was used to specifically visualize extravillous trophoblasts. 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. 5B and E, respectively).

During direct confrontation (no epithelium) extravillous trophoblasts showed strong invasion into the decidual stroma (Fig. 5B). Sampling at different co culture durations showed that first attachment of trophoblastic cell columns and subsequent invasion of HLA-G positive extravillous trophoblast cells took place after 8h of co-culture. A picture of this first attachment is shown figure 5F. Morphological assessment showed that the viability of the tissues is maintained throughout the culture period. There were no visible signs of necrosis even after 140h of culture. Immunohistochemical staining with the proliferative marker Ki67 revealed the presence of proliferative cells after 72h of culture (Fig. 5C, serial section to 5B). Isotype negative control antibodies did not reveal any staining (Fig. 5D). During indirect confrontation (with epithelium) trophoblast cells migrated along the surface of the decidua piece rather than invading deep into the decidua piece (Fig. 5E).

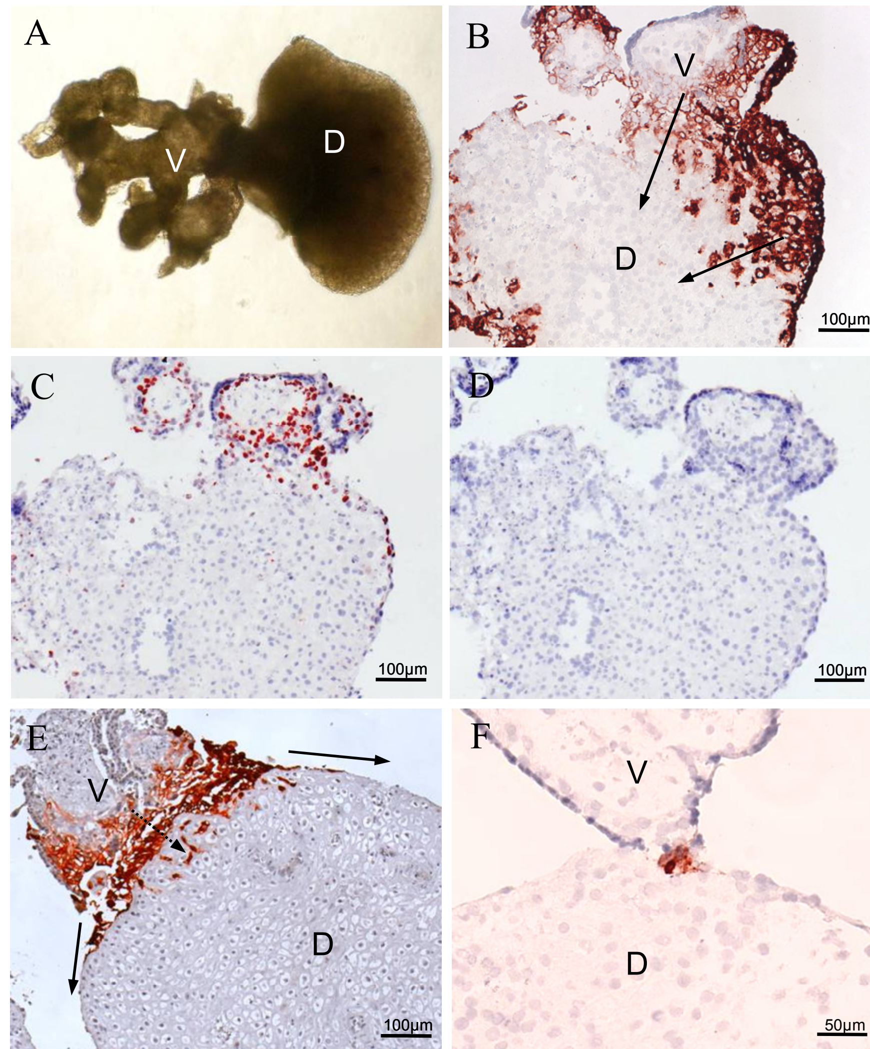

Figure 5: Diverse aspects of confrontation co-culture model systems for invasive extravillous trophoblasts. (A) Image of a confrontation co-culture, the villus firmly attached to the decidua after 72h of confrontation. (B) HLA-G immunostaining of a section of a direct confrontation (GA 8 weeks) after 72h: Strong invasion of extravillous trophoblasts into the decidual stroma can be seen. (C) Serial section to (B) immunostained with the proliferation marker anti-Ki67 shows proliferative cells after 72h culture. (D) Isotype negative control (NC) in a serial section to (B), (C). (E) HLA-G immunostaining of a section of an indirect confrontation (with epithelium, GA 7 weeks), decidua was pre-cultured for 72h prior to confrontation for re-epithelialization. A trophoblastic cell column has formed but invasion into the decidual stroma is only superficial. (F) HLA-G immunostaining of an indirect confrontation, tissues were sectioned after 8h confrontation co-culture. (B-F) Nuclei were counterstained with hemalaun. V = villi, D = decidua (part of this data has been published in Human Reproduction (Moser *et al.*, 2010))

Different approaches were performed to get a three dimensional impression of the confrontation co-cultures. In a simple approach confrontations were serially sectioned and images of serial sections were juxtaposed beside each other. Figure 6 shows HLA-G immunostained images of every tenth cryosection of one single confrontation culture. Juxtaposition of the images enables a three dimensional impression about the invasive

front of the trophoblastic cell column. Invasion and migration of HLA-G positive extravillous trophoblasts can be followed throughout the confrontation. Routinely, every tenth section of a confrontation was assessed for invasive depth of extravillous trophoblasts.

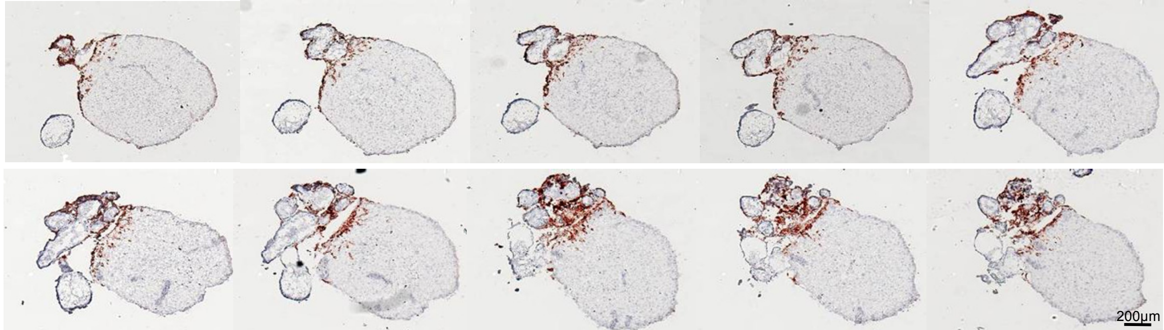

Figure 6: Serial sections of an indirect confrontation (GA 11 weeks) immunostained with antibodies against HLA-G, picture of every tenth section, nuclei were counterstained with hemalaun.

In another approach thick cryosections (~100µm) of the invasive area of direct confrontations were immunofluorescently stained with antibodies against HLA-G. Extravillous trophoblasts (green) invade from the trophoblastic cell column into decidual tissues (Fig. 7). The three dimensional stack in figure 7M is composed of single images like the examples shown in Fig. 7A-L.

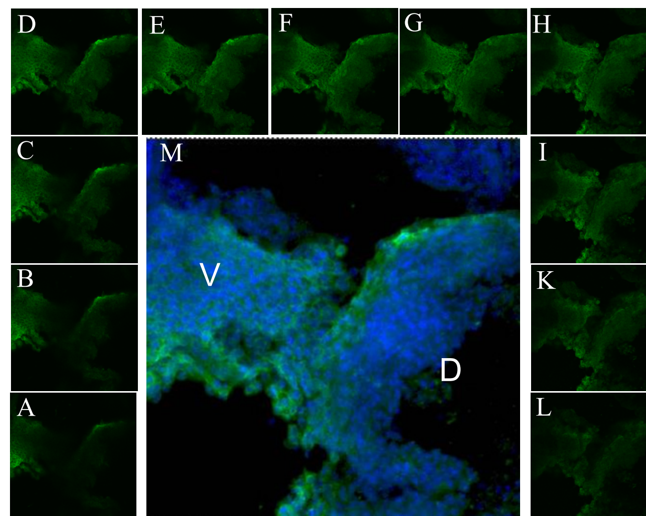

Figure 7: Three dimensional imaging of confrontations with confocal microscopy (A-M) Immunofluorescent stained thick cryosection (~100µm) through the invasive front of a direct confrontation (GA 7 weeks) (green – HLA-G, blue - nuclear counterstain with ToPro3). (A-L) images of every fifth frame stained for HLA-G. (M) shows the stack of all images. V…villi, D… decidua

6.2 Different invasiveness – de-epithelialization

As mentioned above extravillous trophoblasts showed strong invasion into the decidual stroma in direct confrontations (no epithelium) (Fig. 5B), whereas they showed rather migration along the surface of the decidua piece during indirect confrontations (with epithelium) (Fig. 5E). The differences in invasiveness observed in these studies lead to experiments of decidua-denudation /"peeling". Epithelium of re-epithelialized decidua pieces was removed before confrontation.

To influence the observed differing invasiveness during direct versus indirect confrontation co-cultures different approaches to remove the epithelium before indirect confrontations were tested. Epithelium has been removed either by mechanical disruption or by using trypsin. Neither of the two methods removed the epithelium completely. The most efficient way of removing the epithelium uniformly was the use of 0.5% trypsin for 2min. This concentration was determined in preliminary experiments and turned out to be the best compromise between efficient removal of epithelium and minimizing tissue damage. Control confrontations were carried out with untreated re-epithelialized decidua pieces. A schematic representation of the developed protocol is shown in figure 8.

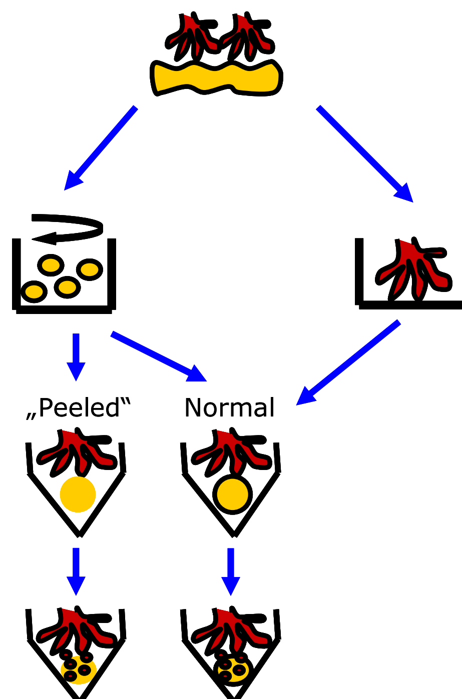

Figure 8: Schematic representation of the experimental procedures for decidua denudation. Epithelium of re-epithelialized decidua pieces was removed before confrontation. "Peeled" decidua pieces were confronted with villi from the same placenta. Control confrontations were carried out with normal re-epithelialized decidua pieces.

For imaging the success of de-epithelialization whole tissue specimens of confrontation co-cultures were fluorescently double stained without prior sectioning. A directly FITC conjugated pan reactive antibody against cytokeratin (clone MNF 116) for labelling trophoblasts as well as glandular and epithelial cells was combined with a directly RPE labelled antibody against HLA Class I (clone W6/32), which stains for all cells except for villous trophoblasts. Stained confrontations were assessed using a confocal microscope. Rests of the cytokeratin positive epithelium can be seen as well as villi attaching to the denudated decidua (Fig. 9A). In the normal indirect confronted control a complete layer of cytokeratin positive cells (green) is covering the decidual stroma (red) (Fig. 9B). The three dimensional stack in figure 9B is composed of single images like the examples shown in Fig. 9C-O.

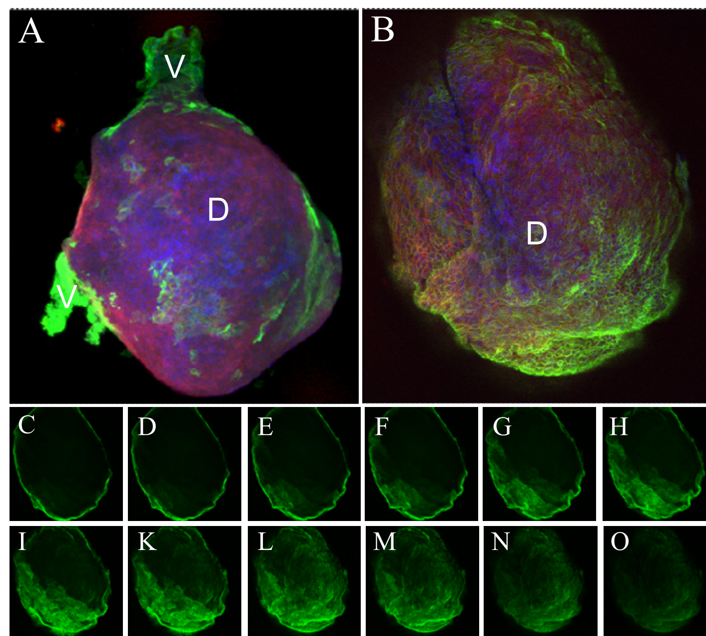

Figure 9: Three dimensional imaging of normal and denuded confrontations with confocal microscopy (A, B) Immunofluorescent staining of whole indirect confrontation (GA 11 weeks) (green – cytokeratin, red – HLA I, blue – nuclear counterstain with ToPro3). (A) Image of complete confrontation, epithelium was partly removed by mechanical disruption before confrontation. The villi attached to the decidua (B) Control: normal indirect confrontation, a dense layer of cytokeratin positive cells (green) is covering the decidual stroma, the villus is not visible in this image section (C-F) images of every tenth frame of B stained for cytokeratin showing the epithelium covering the decidual stroma. V…villi, D…decidua

After preculture the cytokeratin 7 positive replenished epithelium covers the whole surface of the decidua piece (Fig. 10A-). By trypsinization the epithelium could not be removed completely as shown in figure 10B. Attachment of villi to the decidua and formation of trophoblastic cell columns was seen in both experimental setups. Contrary to expectations there was no enhanced invasion in de-epithelialized confrontations.

There was no obvious difference in invasiveness in normal versus de-epithelialized confrontations (Fig. 10C, F). Immunofluorescent double staining with antibodies against cytokeratin 7 for visualizing epithelial cells and entactin for visualizing the basement membrane showed that the basement membrane is still partly conserved after trypsinization (Fig. 10B, E).

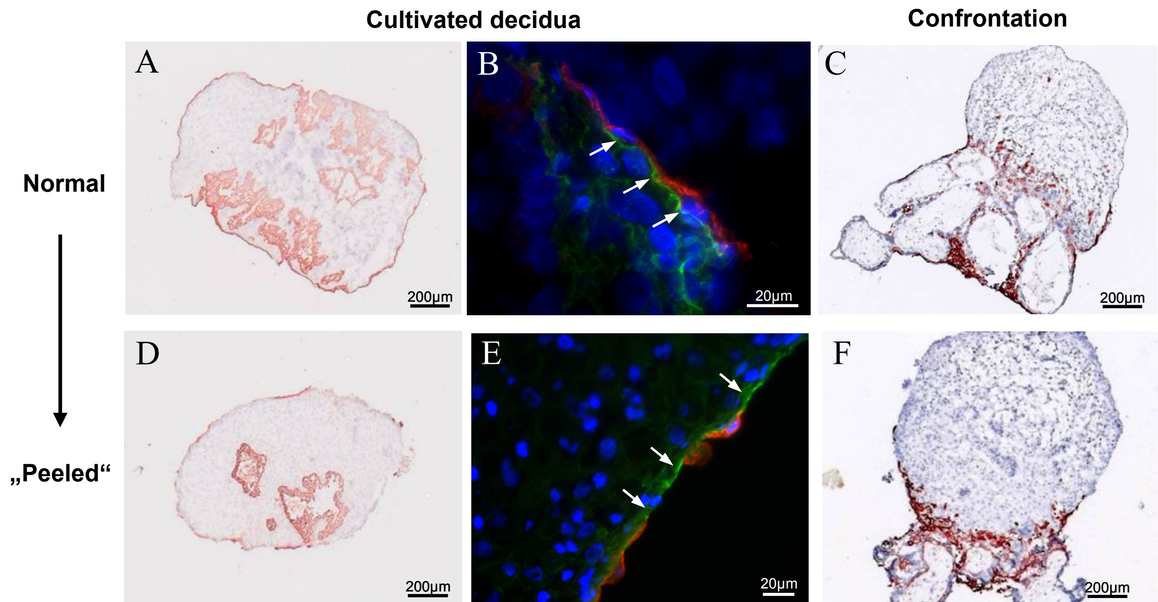

Figure 10: De-epithelialization of decidua pieces and subsequent confrontation (A, D) Cyktokeratin 7 immunostaining of a 72h cultivated decidua piece, (A) cytokeratin 7 positive epithelial cells cover the decidua piece; (D) „peeling“: most of the cytokeratin 7 positive cells were removed. (B, C) Immunofluorescent double staining for visualizing epithelial cells (red - cytokeratin 7) and basement membrane (green – entactin). (B) shows a cultivated decidua piece, epithelial cells are lying on the basement membrane (arrows). (E) After denudation most of the epithelial cells are removed, the basement membrane is still partly intact. (C, F) HLA-G immunostained confrontations, no obvious difference in invasiveness between epithelialized (C) and de-epithelialized confrontation (F). Nuclei were counterstained with hemalaun in A, C, D, F and with DAPI in B, E.

6.3 Different invasiveness – extravillous trophoblasts migrate beyond the epithelium

The recurrence of similar immunohistochemical images of indirect confrontations like in figures 11A, C led to the conclusion that extravillous trophoblasts migrate on the top of the epithelium rather than invading into decidual tissues. Immunofluorescent double staining with antibodies against HLA-G and cytokeratin7 were applied on various sections of indirect confrontations. They showed that extravillous trophoblasts migrate beyond the epithelium rather than on top of it (Fig. 11B, D). Seven placentas were assessed in this respect and in all of the seven placentas extravillous trophoblasts were found to migrate beyond the epithelium.

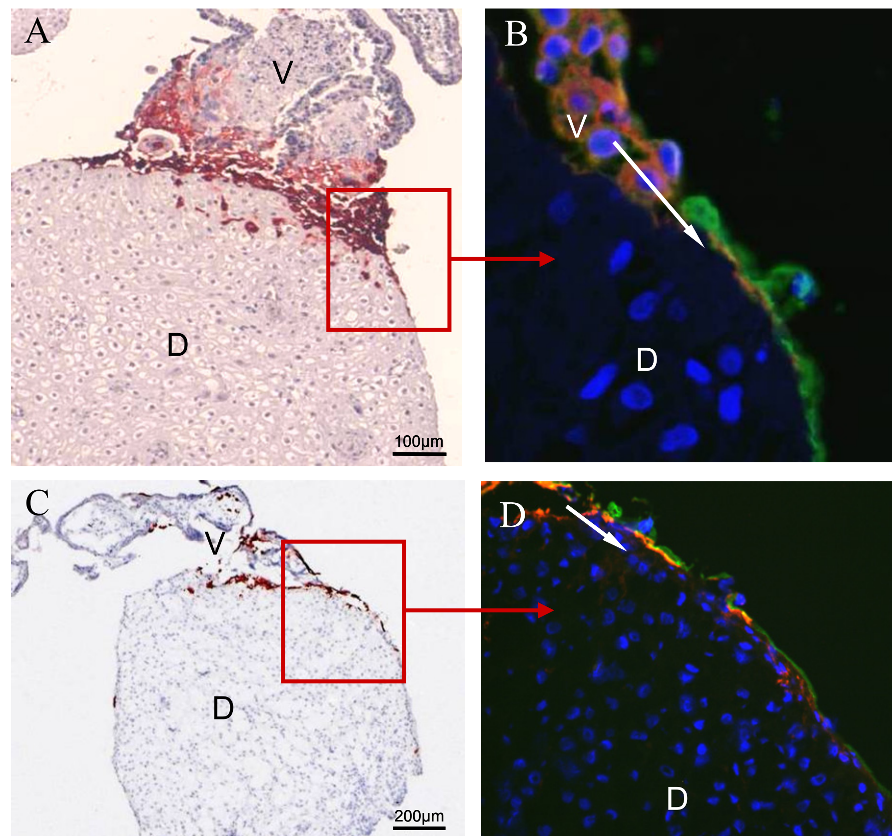

Figure 11: Extravillous trophoblasts migrate beyond the epithelium in indirect confrontations. Sections of indirect confrontations (GA 7 weeks) (A, C) show HLA-G immunostained tissues as an overview of (B, D). (B, D) Immunofluorescent double staining with antibodies against HLA-G (red) and cytokeratin 7 (green) shows extravillous trophoblasts migrating beyond the epithelium (arrow). Nuclei were counterstained with hemalaun (A, C) and DAPI (B, D). V = villi, D = decidua.

6.4 Different invasiveness – oxidative stress

For induction of oxidative stress direct confrontations were stressed either before confrontation or during the whole duration of confrontation co-culture. For separate stressing before confrontation villous and decidual tissues were situated in medium supplemented with 0, 2, and 4mM H_2O_2 before co-culturing in normal medium. An overview of the experimental setup is shown in figure 12.

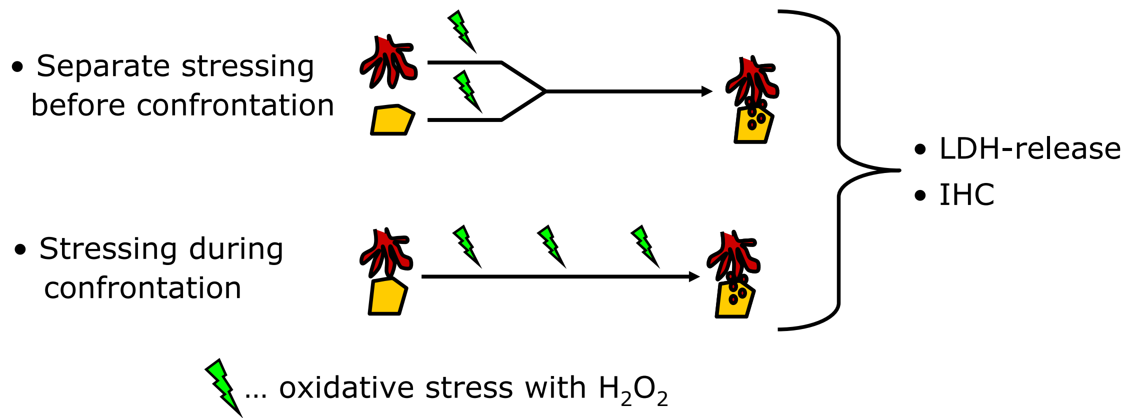

Figure 12: Schematic representation of the experimental procedure for confrontations under oxidative stress. Direct confrontations were either stressed separately before confrontation or stressed during the whole duration of confrontation with different concentrations of H_2O_2. Viability of cells was monitored via LDH-release into the culture media, invasiveness was observed with immunohistochemistry (IHC).

Stressing during the whole duration of confrontation co-culture resulted in tattered tissues and shallow to no adhesion of villous to decidual tissues. Thus, no sectioning and subsequent immunohistochemical assessment of the invasive front was possible.

Stressing for 1h before confrontation turned out to be a condition where attachment of villous to decidual tissues was still ensured. Thus, further experiments were carried out with this protocol. Experiments with three placentas showed a difference in adhesiveness (Tab. 2) but no difference in invasiveness of the villi that attached. With increasing H_2O_2 concentration the adhesiveness decreased.

Table 2: Adhesiveness of confronted tissues decreased with increasing H_2O_2 concentration when tissues were stressed prior to confrontation culture.

Conc H_2O_2	**Number** Adhesion	**%** Adhesion
0 mM	22/26	85
2 mM	16/26	62
4 mM	16/27	59

At every stress condition cell columns were formed and migration as well as invasion of single extravillous trophoblasts into the decidua was seen. Examples of invasion at 0, 2, and 4mM H_2O_2 are demonstrated in figure 13 (Fig. 13A, B and C, respectively). There was no consistent difference in the depth of invasion. The amount of LDH-release into the culture media was not significantly different either (Fig. 14).

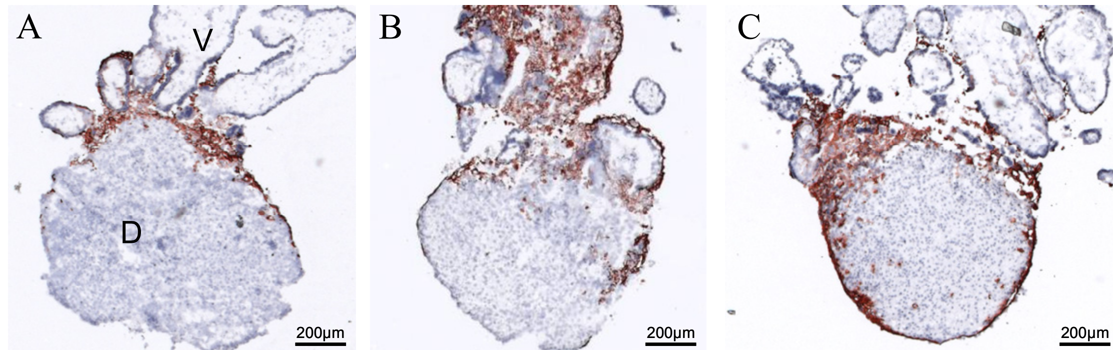

Figure 13: Confrontation and oxidative stress. Examples of HLA-G immunostained sections of direct confrontations, tissues were stressed separately prior to culture with different concentrations of H_2O_2 for one hour and then confronted for 72h. (A) 0mM H_2O_2 (B) 2mM H_2O_2 (C) 4mM H_2O_2. Invasion of extravillous trophoblasts occurs at all H_2O_2-concentrations. Nuclei were counterstained with hemalaun. V = villi, D = decidua (Confrontation co-cultures were processed by Nina Flieser)

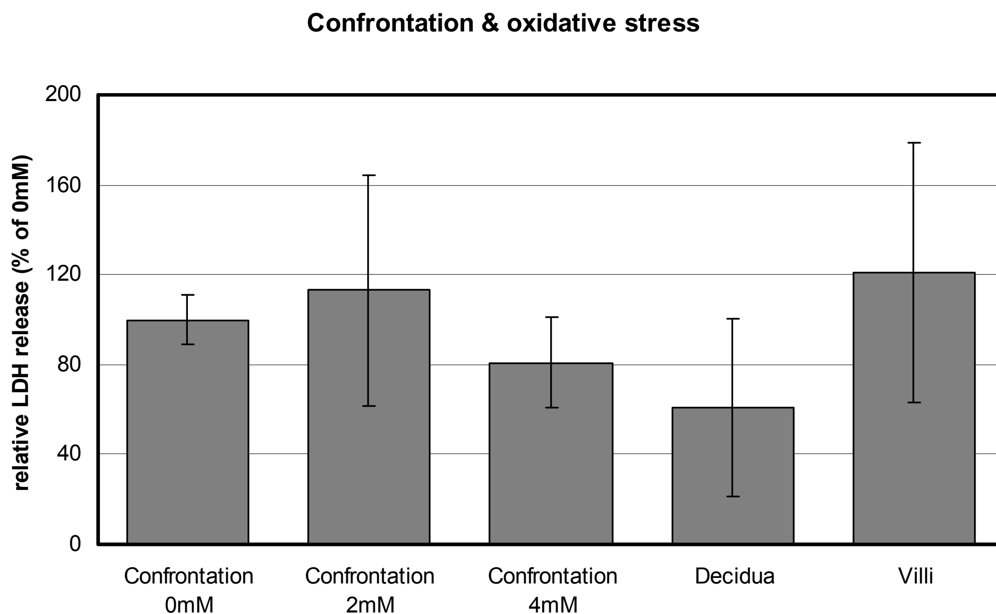

Figure 14: Tissue viability assessed by LDH release in the culture media. Data are normalized to 0mM H_2O_2. Tissue viability is not affected is not affected in the presence of differing H_2O_2-concentrations. The amount of LDH released in the media is not significant according to the student-t test. Data are obtained from three independent experiments. The two bars on the right show LDH release from decidual and villous tissues that were cultured separately without H_2O_2.

6.5 Extravillous trophoblasts and uterine glands

6.5.1 *In situ*

Invasion of extravillous trophoblasts into the decidua basalis was visualized by immunostaining of paraffin sections of first trimester placental tissues with the anti HLA-G antibody 4H84. Extravillous trophoblasts can be found in the decidual stroma and close to uterine glands (Fig. 15A). Extravillous trophoblasts come into close contact with the glandular epithelium and seem to penetrate the basement membrane (Fig. 15B), finally replacing glandular epithelial cells (Fig. 15C).

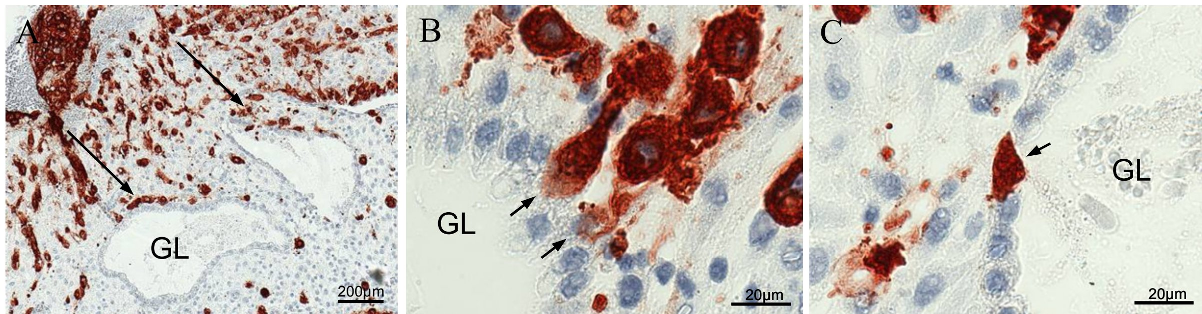

Figure 15: Extravillous trophoblasts reach and invade glands *in situ*. HLA-G immunostaining (antibody 4H84) of a paraffin section of decidua basalis (GA 11 weeks) invaded by extravillous trophoblast. (A) Extravillous trophoblasts invade from cell columns towards uterine glands (arrows). (B) Extravillous trophoblast cells (arrows) seem to penetrate the glandular basement membrane from the stromal side. (C) An extravillous trophoblast (arrow) has crossed the glandular basement membrane and has replaced a glandular epithelial cell. Nuclei were counterstained with hemalaun. GL = glandular lumen. (This data has been published in Human Reproduction (Moser *et al.*, 2010))

Extravillous trophoblasts can be found in close vicinity as well as inside the lumen of uterine glands throughout the first trimester (Fig. 16). Paraffin sections of invaded first trimester decidua basalis were immunohistochemically double stained with antibodies against HLA-G and cytokeratin 7. Figure 16 shows that extravillous trophoblasts are situated nearby uterine glands and replace glandular epithelial cells during the gestational period of 6 to 11 weeks of pregnancy (Fig. 16C, D, E, F, H, I, K). In some cases extravillous trophoblasts even infiltrate the uterine lumen (Fig. 16B, G). In every first trimester decidual section that was double stained for HLA-G and cytokeratin 7 extravillous trophoblasts were found in and/or in very close vicinity of uterine glands. In the invaded parts of sections of decidua basalis 55% ± 7% (mean ± SEM; n = 10, range 6 to 11 weeks) of glandular cross sections were associated with or infiltrated by extravillous trophoblasts.

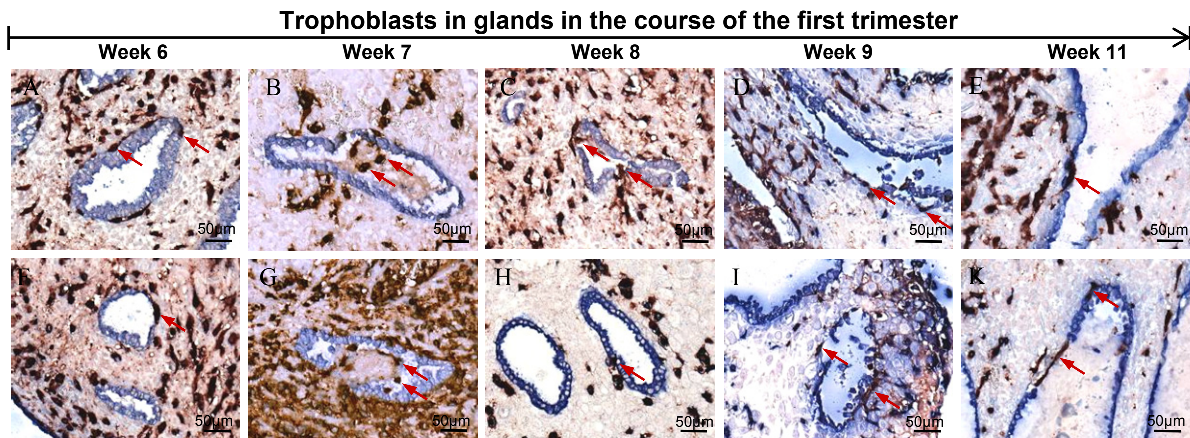

Figure 16: Extravillous trophoblasts replace glandular cells during the first trimester. (A-K) Immunohistochemical double staining for HLA-G in red and cytokeratin 7 in blue on 10 paraffin sections of decidua basalis to visualize trophoblasts nearby and inside uterine glands. Extravillous trophoblasts appear in dark violet / brown, epithelial cells in blue. Red arrows indicate extravillous trophoblasts nearby and in uterine glands. (A) Extravillous trophoblasts nearby a uterine gland at week 6. (C-F, H-K) Extravillous trophoblasts have replaced glandular epithelial cells. (B, G) Extravillous trophoblasts have replaced uterine epithelial cells and some of them have also infiltrated into the glandular lumen. From week 10 no specimen was available. No nuclear counterstain. (This data has been published in Human Reproduction (Moser *et al.*, 2010))

6.5.2 In vitro

Also in our model systems invasive extravillous trophoblasts can be found in close vicinity to uterine glands (Fig. 17). Immunofluorescent double staining using antibodies against HLA-G to detect extravillous trophoblast (red – yellow) and cytokeratin to detect trophoblasts and glandular epithelial cells (yellow - green) were applied on sections from direct and indirect confrontations (Fig. 17B, C). During indirect confrontation extravillous trophoblasts migrate along the surface of the re-epithelialized decidua piece and in direction towards the glandular lumen (Fig. 17B). During direct confrontation extravillous trophoblasts 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. 17C, circle). The localization and putative movement of extravillous trophoblasts was visualized in schematic drawings (Fig. 17D, E).

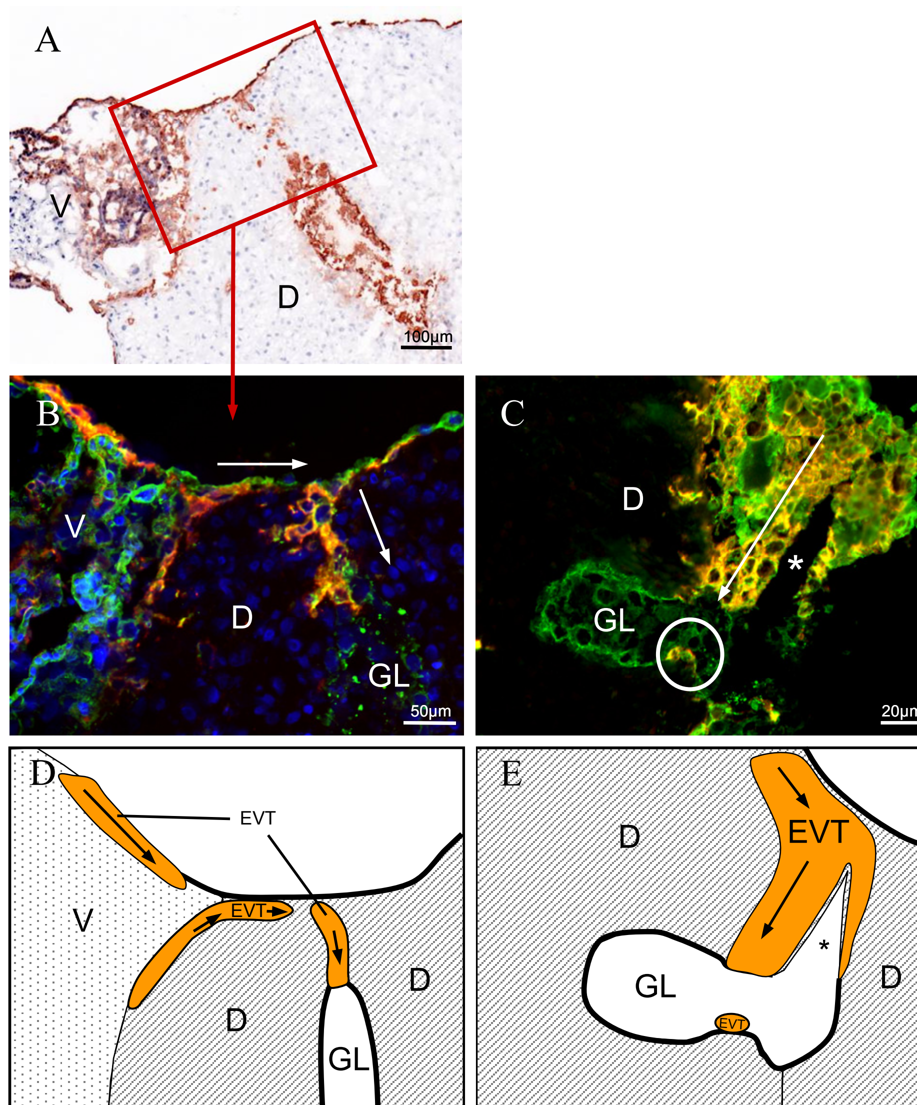

Figure 17: Extravillous trophoblasts are approaching uterine glands in direct and indirect confrontation assays. (A) Overview of an indirect confrontation (GA 6 weeks) with immunostaining against cytokeratin staining trophoblast and epithelium. (B, C) Immunofluorescent double staining using antibodies against HLA-G to visualize extravillous trophoblast (red – yellow) and cytokeratin 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 glandular lumen (marked by the white arrows). (C) During direct confrontation (GA 7 weeks) extravillous trophoblasts 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, E) Schematic representation of (B) and (C) to depict the different cell types and routes of invasion. Nuclei were counterstained with DAPI. EVT = extravillous trophoblast, V = villi, D = decidua, GL = glandular lumen. (This data has been published in Human Reproduction (Moser *et al.*, 2010))

Staining of additional direct confrontations showed extravillous trophoblasts lying between glandular epithelium and glandular basement membrane (Fig. 18). Double immunofluorescent staining for HLA-G and cytokeratin 7 revealed the presence of

extravillous trophoblasts beneath glandular and uterine epithelial cells (Fig. 18C, D). Double immunofluorescence for the basement membrane marker entactin and HLA-G on neighboring serial sections demonstrated that extravillous trophoblasts were lying on top of the basement membrane (luminal side) (Fig. 18E, F).

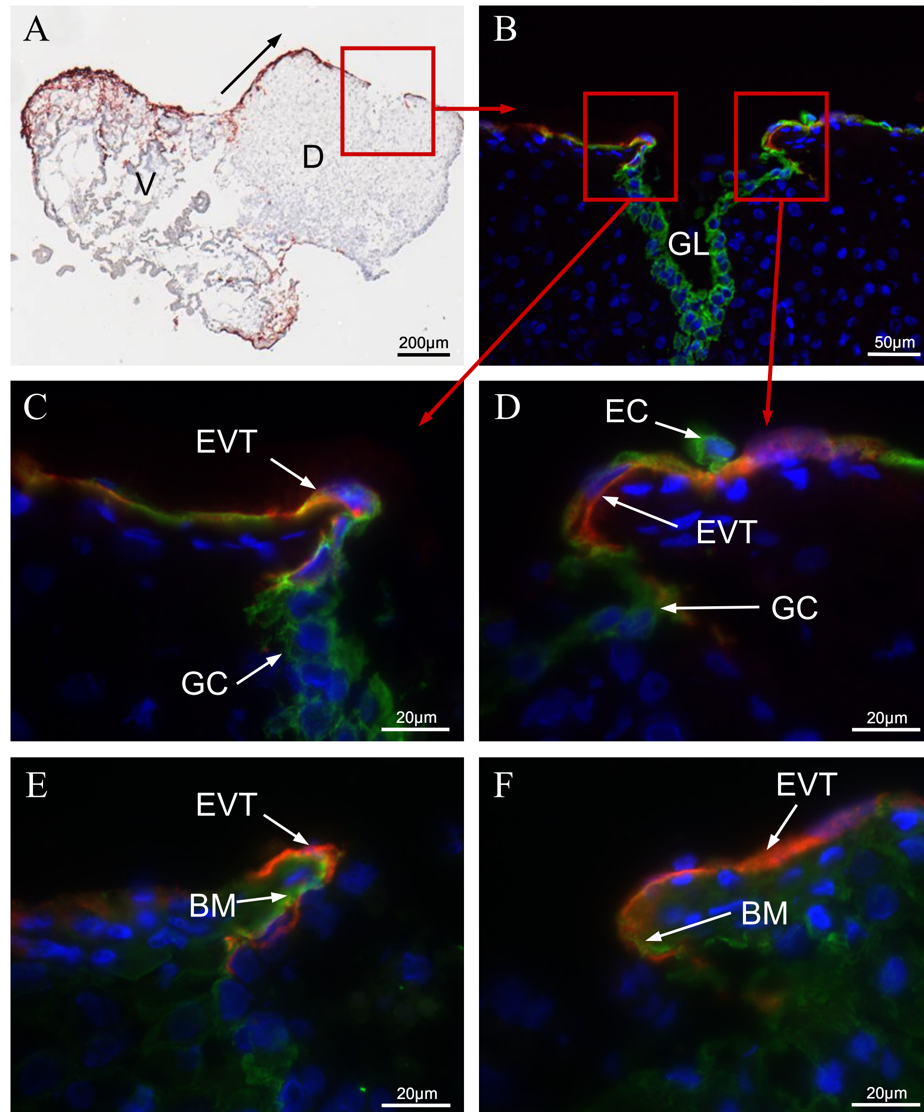

Figure 18: Direct contact between extravillous trophoblast and glandular epithelial cells. (A) Overview of a direct confrontation (GA 7 weeks) with immunostaining against HLA-G. Extravillous trophoblasts migrate from the villus along the surface of the decidua (black arrow). (B, C, D) Immunofluorescent double staining for cytokeratin 7 and HLA-G shows glands and epithelium (green) and extravillous trophoblasts (red – yellow). Trophoblasts are located beyond glandular and uterine epithelial cells. (E, 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 top of the basement membrane between the membrane and the glandular epithelial cells. Nuclei were counterstained with DAPI. V = villi, D = decidua, GL = glandular lumen, EVT = extravillous trophoblasts, BM = basement membrane, GC = glandular cell, EC = epithelial cell. (This data has been published in Human Reproduction (Moser *et al.*, 2010))

The occurrence of extravillous trophoblasts lying between basement membrane and epithelial cells was also clearly confirmed by immunofluorescent triple staining. For this purpose a neighboring serial section to figure 18A was stained with antibodies against HLA-G, cytokeratin7 and entactin simultaneously (Fig. 19B, C).

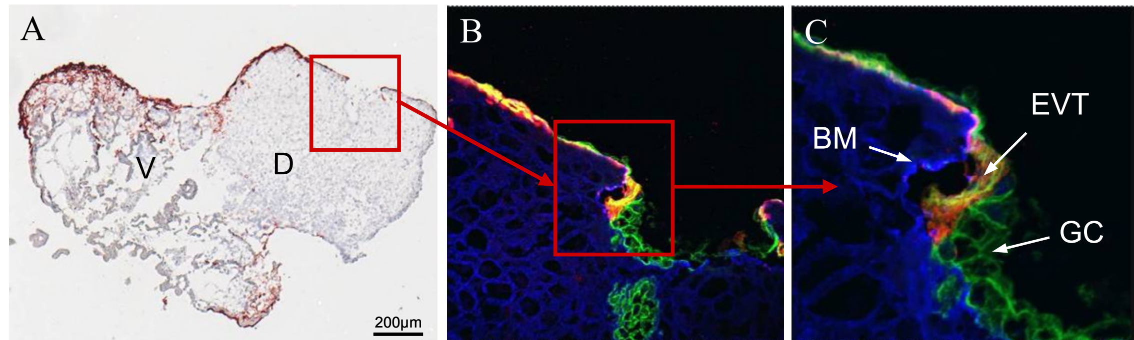

Figure 19: Extravillous trophoblasts migrate between epithelium and basement membrane (A) Overview of a direct confrontation (GA 7 weeks) with immunostaining against HLA-G. Extravillous trophoblasts migrate from the villus along the surface of the decidua (black arrow). (B, C) Immunofluorescent triple staining for cytokeratin 7, HLA-G and entactin shows glands and epithelium (green), extravillous trophoblasts (red – yellow) and the basement membrane (blue). Trophoblasts are located between basement membrane and glandular epithelial cells. (A) Nuclei were counterstained with hemalaun. (B, C) No nuclear counterstain. BM = basement membrane, GC = glandular cell, EVT = extravillous trophoblast.

7 Discussion

7.1 Confrontation co-culture

7.1.1 Own experience

In this thesis novel *in vitro* double tissue confrontation co-culture model systems of decidual tissues with villous explants to mimic extravillous trophoblast invasion during placentation are presented. Such *in vitro* model systems display useful tools for studying trophoblast invasion, although they have certain limitations, which is true for each artificial system.

Sometimes, the comparability between the experiments turned out to be difficult due to variability between the placentas. Supply with placentas was occasionally problematic, sometimes there were several weeks without confrontation experiments because the tissues were not suitable for confrontation co-culture (e.g.: no villi in the sample, no decidua in the sample, decidua only disrupted and not compact, etc). The whole tissue processing for the confrontation co-culture experiments is laborious; the accomplishment of an experiment including cryosectioning, staining and subsequent analysis takes at least two weeks. Thus, the developed assays are not suitable for producing “quick results”.

Beyond the rare successful photographs shown in this thesis there is an immense number of sections without explanatory power. Fortunately, several approaches for getting a three-dimensional impression of the invasive front were carried out successfully (see Fig. 6, 7 and 9). The effects of adhesiveness / invasiveness seem to be more dependent on the properties of the individual placenta than on the factors changed in the experimental setup (like denudation, oxidative stress). This may be due to the fact that manipulation of specific cells in such a system is nearly impossible without affecting other cell types as well. However, beside all these 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.

7.1.2 Confrontation co-cultures in literature

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 (Babawale *et al.*, 2002; Dunk *et al.*, 2003; Vicovac *et al.*, 1995). Placing villous explants on collagen I allows 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 remodeling can only be achieved by 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 (Dunk *et al.*, 2003; Vicovac *et al.*, 1995). 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. Factors possibly influencing trophoblast invasion were minimized as much as possible in the novel confrontation co-culture systems described here. 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 earlier (Helige *et al.*, 2008; Helige *et al.*, 2001), but to the best of my knowledge hormones have never been added to decidual – villous co-cultures. Since the presence of progesterone and 17-β-estradiol is crucial to maintain decidual tissues, these hormones are present in the confrontation co-cultures to best mimic the *in vivo* situation. Similarly, the confrontation co-cultures are carried out at ambient air, since invading extravillous trophoblasts experience high oxygen concentrations different to villous trophoblast in the first trimester of pregnancy.

Re-epithelialization of cultured decidua pieces has already been described by other authors (Helige *et al.*, 2008; Helige *et al.*, 2001; Vicovac *et al.*, 1995). Cells were observed to migrate out from the necks of endometrial glands on top of the surface of

the decidual stroma where they achieved a more flattened morphology (Vicovac *et al.*, 1995). In my confrontation systems the cultured decidua pieces follow the same route of re-epithelialization (see Fig. 4).

So far the general picture of trophoblast - decidua interactions is that motile extravillous trophoblast cells actively invade into an inflexible, stiff and settled matrix of decidual cells. Secretions by the decidual cells like protease inhibitors may inhibit and/or control the invasive behaviour of the extravillous trophoblasts. Recently, it was demonstrated that endometrial stromal cells have an invasive phenotype which is induced upon decidualization and further enhanced by close contact with, and/or factors secreted from trophoblastic cells (Gellersen *et al.*, 2010). It was found that not only the fetally derived trophoblast cells, but also the maternally derived decidual cells display invasive potential beside already earlier described migratory potential. Thus, the authors suggest that decidual cells are equipped to actively support the movement of extravillous trophoblasts into the decidual layer by encircling them. They propose that the maternal decidua might play a more active role and has a much greater contribution in encapsulating the early conceptus and supporting the subsequent trophoblast invasion than hitherto recognized. Although these conclusions were drawn from cell culture experiments they give reason for reconsideration of events and activities of cells in a complex tissue.

7.2 Different invasiveness of extravillous trophoblasts

7.2.1 Oxidative stress

Invasiveness of extravillous trophoblasts is varying between the model systems. In an approach to actively influence this invasiveness, oxidative stress was induced in the model system. Oxidative stress is considered to play a role in multiple physiological processes from oocyte maturation to fertilization and embryo development. There is evidence that oxidative stress is contributing to conditions such as abortion, pre-eclampsia, hydatidiform mole, fetal embryopathies, preterm labor, and gestational diabetes, all of which lead to an immense burden of maternal and fetal morbidity and mortality. Reactive oxygen species can entail to infertility problems and a variety of female reproductive disorders. Free radicals hold important physiological functions in the female reproductive tract, while at the same time excessive free radicals may induce female reproductive tract pathologies (Agarwal *et al.*, 2005).

Two major types of free radical species are known; reactive oxygen species (ROS) and reactive nitrogen species (NOS); the three major types of ROS are superoxide, hydrogen peroxide and hydroxyl. ROS are involved in more than 100 diseases; and are known to play a physiological as well as a pathological role in the female reproductive tract. The balance is sustained by the presence of adequate amounts of antioxidants. A detailed overview about the role of oxidative stress in pregnancy can be found in Agarwal *et al.* (Agarwal *et al.*, 2005).
In the confrontation co-culture system hydrogen peroxide was used to induce oxidative stress. Stressing during the whole duration of confrontation co-culture resulted in tattered tissues and shallow to no adhesion. Hence, in this experimental approach it was not possible to section and stain tissues and subsequently evaluate invasiveness. Evaluation of putative effects of oxidative stress on invasiveness was possible when oxidative stress was induced in tissues separately prior to confrontation co-culture in normal media. Thereby, cell columns were formed and migration as well as invasion of single extravillous trophoblasts into the decidua was seen at every stress condition (Fig. 13). Increasing concentrations of hydrogen peroxide resulted in decreased adhesion of villi to decidua. More than half of the tissues still adhered after being encountered even to 4mM H_2O_2. The tissues obviously survive if exposed to hydrogen peroxide and still adhere. During the subsequent confrontation the cells were not stressed anymore. This may explain that there was a differing adhesiveness, but no difference in invasiveness after confrontation co-culture. So there may be a correlation between oxidative stress and adhesion, but since there is no stress during co-culture there may be no effect anymore on invasiveness or viability of cells.

7.2.2 Extravillous trophoblasts and uterine epithelium

With the model systems it was possible to show that the invasiveness of extravillous trophoblasts depends on the presence or absence of a decidual epithelium. In the absence of an epithelium, i.e. in direct contact of trophoblasts and decidual stroma, deep invasion into the decidual pieces is obvious (see Fig. 5B and Fig. 7). In indirect confrontation assays, when re-epithelialized decidual pieces are used for confrontation, extravillous trophoblasts migrate along the surface of the decidua pieces (see Fig 4B and Fig. 6). Interestingly, the extravillous trophoblasts do not migrate on top of the epithelium as assumed by Vicovac *et al* (Vicovac *et al.*, 1995). From immunofluorescent double staining in indirect confrontation co-culture assays it was

demonstrated that extravillous trophoblasts rather migrate beyond the epithelium on top of the basement membrane (Fig. 11). This fits together with the results from the denudation approaches. Contrary to expectations there was no enhanced invasion in de-epithelialized confrontations. Later staining revealed that the basement membrane was still intact after removal of the covering epithelium (see Fig. 10B and E). Thus, it seems as if the extravillous trophoblasts do not invade through the basement membrane from the epithelial side. Rather, it seems as if extravillous trophoblasts migrate between epithelial cells and epithelial basement membrane (see Fig. 18 and 19). This is true for the luminal epithelium as well as for the glandular epithelium. Such a localization of extravillous trophoblasts can also be seen *in situ* (see Fig. 15A), where trophoblasts are located in direct vicinity underneath the glandular epithelial cells. However, so far there is no proof that trophoblasts are lying between epithelial cells and basement membrane *in situ*.

7.3 Extravillous trophoblasts and uterine glands

The presented data also demonstrates that extravillous trophoblasts can be found nearby the glandular epithelium, *in situ* and *in vitro*. This finding is in accordance with data from Demir *et al.* showing cytokeratin positive extravillous trophoblasts in direct vicinity to decidual glands in placental tissues between days 18 and 41 post conception (Demir *et al.*, 2002). Hempstock *et al.* performed studies on *in situ* specimens of early gestational material and posed that many invading trophoblasts, macrophages and NK 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 my knowledge the presence of extravillous trophoblasts lying inside glands has never been described in literature. This may be due to the fact that earlier groups did not use specific staining to differentiate between extravillous trophoblasts and glandular epithelial cells. It is very difficult to identify trophoblasts at or in glands in H&E sections or in sections immunohistochemically stained for cytokeratin 7 since both cell types (trophoblasts and glandular cells) are stained with anti-cytokeratin antibodies. Only using a double staining for cytokeratin and HLA-G clearly reveals the presence of extravillous trophoblast 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 number of glands penetrated by trophoblasts even at up to 11 weeks of gestation, other mechanisms need to come into

play to explain the high number of glandular cross sections that have been found to contain extravillous trophoblasts.

Burton *et al.* showed that the secretion products of the uterine glands are released into the intervillous space but provided no explanation on how the lumen of the uterine glands may be connected with the intervillous space (Burton *et al.*, 2002). In contrast to earlier studies I had the possibility to apply specific antibodies to clearly distinguish between invasive extravillous trophoblasts and glandular cells. I could show infiltration of extravillous trophoblasts 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. The data indicates that extravillous trophoblasts 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 figure 20. Clear evidence of extravillous trophoblasts even lying in the glandular lumen is demonstrated in figures 16B, 17B and 17C.

According to the definition in Benirschke *et al.* there are different phenotypes of invasive extravillous trophoblasts, the interstitial trophoblast with its subtypes (Kemp *et al.*, 2002) and endovascular trophoblast (Frank and Kaufmann, 2006). Referring to the presented data a new route of trophoblast invasion is suggested; “endoglandular trophoblast”. Endoglandular trophoblasts were found repeatedly nearby uterine glands, replacing the glandular epithelium or even present in the glandular lumen. This could be shown in every specimen assessed throughout the first trimester of pregnancy. The invasion into glands is not a rare event; this is indicated by the series of images with endoglandular trophoblasts in figure 16 as well as the approach to count endoglandular trophoblasts in glandular cross sections. However, this simple quantification approach from singular *in situ* sections without a clear orientation does not meet the requirements of a valid quantification approach. Hence, a respective study including more and larger tissue samples is required.

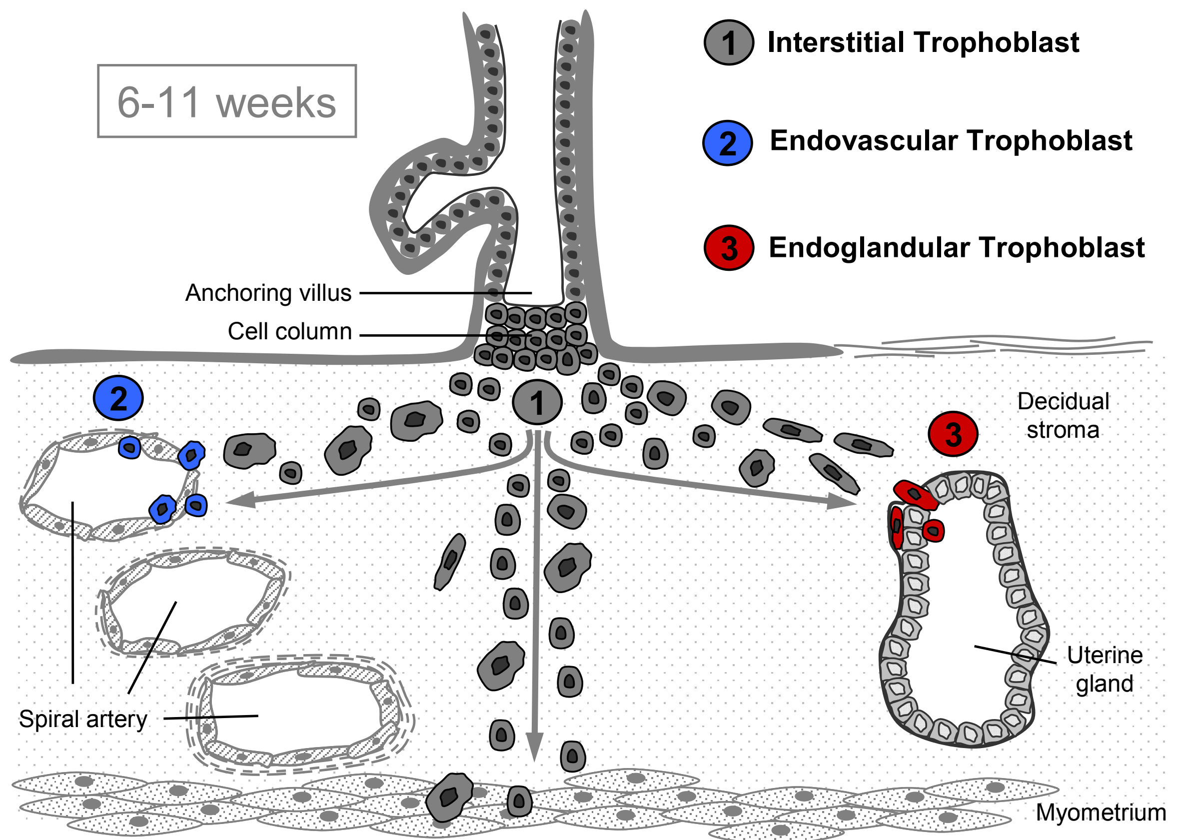

Figure 20: Scheme of trophoblast invasion of the decidua including uterine glands. Extravillous trophoblasts originate from cell columns of anchoring villi. During the first trimester of pregnancy extravillous trophoblasts invade through the decidual interstitium (1) and follow different routes of invasion. 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 to the glandular lumen. (This scheme has been published in Human Reproduction (Moser *et al.*, 2010))

Invasion of spiral arteries is a directed and controlled process. Starting from interstitial trophoblast a subset of extravillous trophoblasts 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 vessels' media. Endoglandular invasion displays aspects similar to endovascular invasion; extravillous trophoblasts 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 extravillous trophoblasts, for example uterine veins. Whether or not pathologies derive from failure of invading decidual glands has not been addressed yet in any study.
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. These novel confrontation co-culture model systems enable at least a partial imitation of the changes in maternal tissues occurring during trophoblast invasion and placentation *in vivo*.

8 Outlook

Establishment of the uteroplacental blood flow starts with the end of the first trimester. Thus, the embryonic and maternal tissues are exposed to different oxygen concentrations in the first twelve weeks of pregnancy. Before opening of the spiral arteries into the intervillous space villi are maintained in an oxygen concentration of 2-3%. After opening of the spiral arteries the oxygen concentration rises to about 8%, whereas the maternal tissues are continuously exposed to about 15% oxygen. Until now these gradients and changes in oxygen concentration could not be mimicked *in vitro*. Thus, a new confrontation co-culture chamber is in development where confrontation co-culture of decidua and villi under separate oxygen concentrations will be possible. In cooperation with a plastic processing company a plan for the chamber has already been designed. Villous and decidual tissues will be fixed together and medium (conditioned to an appropriate oxygen concentration) will be pumped separately through the upper and lower part of the chamber (Fig. 21).

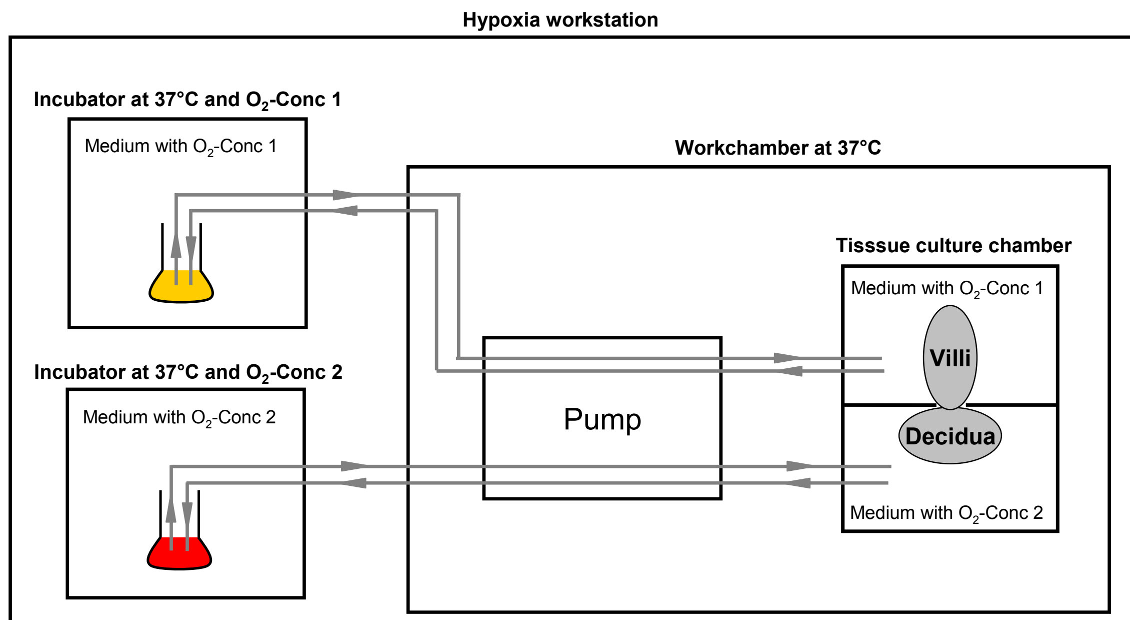

Figure 21: Scheme for a future culture system where confrontation co-culture of decidua and villi under separate oxygen concentrations will be possible. The whole system will be set up in a hypoxia workstation. Medium with O_2-concentration 1 will be pumped through the upper part of the chamber where villous explants are housed. The lower part of the chamber will be flooded with medium with O_2-concentration 2 that will reach the decidual tissues.

9 Abbreviations

BM – basement membrane

D – decidua

DAPI – 4,6 diamidino–2-phenylindoledihydrochloride

DMEM – Dulbecco's modified eagle medium

EC – epithelial cell

EDTA – ethylenediaminetetraacetic acid

EVT – extravillous trophoblast

FITC – fluorescein-5-isothiocyanat

GA – gestational age

GC – glandular cell

GL – glandular lumen

H&E – hemalaun-eosin

HBSS – hank’s buffered salt solution

HLA – human leukocyte antigen

IgG – immunoglobulin G

IHS – immunohistochemistry

LDH – Lactate dehydrogenase

NOS – reactive nitrogen species

PBS – phosphate buffered saline

ROS – reactive oxygen species

SEM – standard error of the mean

TBS – Tris buffered saline

TBS-T – Tris buffered saline, supplemented with Tween

V – villi

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11 Publications

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12 Materials

12.1 Materials for confrontation co-culture

Article	*Cat. No*	*Company*
Spinner flask 25ml	--	GTW, Vienna, Austria
Various tweezers	--	Lactan, Graz, Austria
Präpariernadelhalter nach Kolle	HWO-02017	Lactan, Graz, Austria
Single use scalpels	L4A9.409809	Bartelt, Graz, Austria
Petri dish 94x16mm	633181	Greiner Bio One, Kremsmünster, Austria
Petri dish 3.5x1cm	627161	Greiner Bio One, Kremsmünster, Austria
1000µl pipet tips, shortened before autoclaving, for transfer of tissues	0030000.919	Eppendorf, Hamburg, Germany
2ml reaction tubes, perforated before autoclaving	0030120.094	Eppendorf, Hamburg, Germany
DMEM high glucose w/o L-glutamine, w sodium pyruvate	E15-011	PAA, Pasching, Austria
HBSS w/o Ca&Mg w/o phenole red	H15-009	PAA, Pasching, Austria
L-Glutamine 200mM	M11-004	PAA, Pasching, Austria
Amphotericin B	P11-001	PAA, Pasching, Austria
Penicillin / Streptomycin	P11-010	PAA, Pasching, Austria
Progesterone	962670	Schering, Berlin, Germany
17-β-Estradiol	317347	Schering, Berlin, Germany
10x Tryspin-EDTA	L11-003	PAA, Pasching, Austria
Reaction tube racks with lid, autoclavable	T219.1	Lactan, Graz, Austria
TissueTek®	4583	Sakura Finetek Inc, Torrance, USA

12.2 Materials for immunohistochemistry / immunofluorescence

Article	*Cat. No*	*Company*
Superfrost plus glass slides	J1800AMNZ	Menzel, Braunschweig, Germany
Epitope retrieval solution pH 9	DEPP-9	Eubio, Vienna, Austria
Tissue-Tek® O.C.T.TM Compound	4583	Sakura Finetek Europe B.V.
DakoCytomation Pen (PAP pen)	S 2002	DAKO, Vienna, Austria
Antibody diluent	S3022	DAKO, Vienna, Austria
Hydrogen Peroxide Block	TA-060-HP	LabVision, CA, USA
Ultra V Block	TA-60-UB	LabVision, CA, USA
Primary Antibody Enhancer	TL-060-PB	LabVision, CA, USA
HRP Polymer	TL-060-PH	LabVision, CA, USA
Multivision Polymer detection system	TL-012-MHRA	Eubio, Vienna, Austria
AEC (3-Amino-9-ethylcarbazol)	TA-125-SA	LabVision, CA, USA
Mayer's hemalaun	1.09249	Merck, Darmstadt, Germany
Kaisers glycerol gelatine	1.09242.0100	Merck, Darmstadt, Germany
ProLong Gold Antifade reagent	P36930	Invitrogen, Lofer, Austria
4,6 diamidino -2-phenylindoledihydrochloride (DAPI)	D1306	Invitrogen, Lofer, Austria
ToPro3 iodide	T3605	Invitrogen, Lofer, Austria
O.C.T compound tissue freezing medium, TissueTek®	4583	Sakura Finetek Inc, Torrance, USA
Cover glass assistant 32x24 mm	No. 990	Roth/Lactan, Graz, Austria

12.3 Devices

Device	*Name*	*Company*
Decloaking chamber	DC2002	Sanova, Vienna, Austria
Humidified incubator	Cytoperm2	Heraeus Instruments GmbH, Hanau, Germany
Centrifuge	Function Line Labofuge 400R	Heraeus Instruments GmbH, Hanau, Germany
Laminar flow	ClanLAF VFR1206	Damm, Fredensborg, Denmark
Horizontal laminar air flow work station	Lamin Air H36	Holten, Allerød, Denmark
Magnetic stirrer for the incubator	Telesystem 06.40 and Telemodul 40C	H+P Labortechnik AG, Oberschleißheim, Germany
Cryocut	HM 560 V	Microm, Walldorf, Germany
LSM	Leica SP2	Leica Lasertechnik GmbH, Heidelberg, Germany
Photo documentation of immunohistochemistry	Axiophot	Zeiss, Jena, Germany
Paraffin embedding	Shandon Citadel	Thermo Scientific, USA

12.4 Buffers & Solutions

Ammonium water

2.5ml ammoniac (25%)

ad 1l aqua bidest

10x PBS

Na_2HPO_4; H_20x12; 81mM

NaCl; 1.54M

KH_2PO_4; 19mM

with aqua dest. ad 1l

adjust pH to 7.2

10x TBS

1.5mM NaCl

0.5 M Tris pH8.0

with aqua dest. ad 1l

adjust pH to 7.4

1x TBS-T

1xTBS

0.05% Tween

13 Protocols

13.1 Tissue processing for indirect confrontation co-culture

- Prewarm media to 37°C, place placenta in a Petri dish with preheated media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2mm pieces, cut villi into pieces of corresponding size (15-20mg)
- Fill a spinner flask with 25ml media
- Transfer decidua pieces into spinner flask with shortened pipette tip
- Preculture of decidua at 37°C, 5% CO_2 on a magnetic stirrer at ~130rpm for 72h
- Change media in spinner flasks after 48h
- Place villi in Petri dish with 20ml media
- Preculture of villi at 37°C, 2.5% O_2 and 5% CO_2 for 72h in a hypoxic workstation (cover Petri dish with aluminium foil if used; hypoxic workstation is translucent)
- Start confrontation co-culture after 72h of preculture
- Transfer decidua pieces and villi into small Petri dishes with fresh media
- Fill perforated 2ml reaction tubes with 500µl media and place in a tube rack with lid
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua or villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze at -80°C until sectioning

13.2 Tissue processing for direct confrontation co-culture

- Prewarm media to 37°C, place placenta in a Petri dish with prewarmed media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2mm pieces, cut villi into pieces of corresponding size (15-20mg)
- Transfer decidua pieces and villi into small Petri dishes with fresh media
- Fill perforated 2ml reaction tubes with 500µl media and place in a tube rack with lid
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua and villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze at -80°C until sectioning

13.3 Immunohistochemistry for cryosections	
• Thaw cryosections in glass cuvette	10 min
• Air-dry	10 min
• Fixation in acetone at room temperature	5 min
• Air-dry	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash in 1 x PBS	5 min
• Incubate with Hydrogen Peroxide Block	12 min
• Wash three times in 1 x PBS	2 min, each
• Incubate with Ultra V Block supplemented with 10% human AB-serum	8 min
• Tap off UV-Block and apply primary antibody-solution, incubate at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply Primary Antibody Enhancer, incubate at room temperature	10 min
• Wash three times in 1 x PBS	2 min, each
• Apply HRP-Polymer, incubate at room temperature	15 min
• Wash three times in 1 x PBS	2 min, each
• Apply AEC and observe regularly for staining intensity	6 – 10 min
• Wash three times in deionized water	2 min, each
• Counterstain in hemalaun	3 min
• Rinse three times in deionized water	
• Blueing in ammonium water	1 min
• Rinse three times in deionized water	
• Tap off excessive water	
• Mount with Kaiser´s glycerol gelatine	

13.4 Immunohistochemistry for paraffine sections	
• Deparaffinize slides through a series of graded alcohols	
• Xylene 1	10 min
• Xyvlene 2	10 min
• Xylene / 100% Ethanol	5 min
• 100% Ethanol	3 min
• 96% Ethanol	3 min
• 96% Ethanol	3 min
• 70% Ethanol	3 min
• Deionized water	5 min
• Arrange slides in plastic cuvette filled with antigen retrieval solution	
• Heat induced antigen retrieval in a pressure cooker at 120°C	7 min
• Cool down at room temperature	20 min
• Deionized water	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash three times in 1 x TBS-T	2 min, each
• Incubate with Hydrogen Peroxide Block	12 min
• Wash three times in 1 x TBS-T	2 min, each
• Incubate with Ultra V Block	8 min
• Tap off UV-Block and apply primary antibody-solution, incubate at room temperature	30 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply Primary Antibody Enhancer, incubate at room temperature	10 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply HRP-Polymer, incubate at room temperature	15 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply AEC and observe regularly for staining intensity	6 – 10 min
• Wash three times in deionized water	2 min, each
• Counterstain in hemalaun	3 min
• Rinse three times in deionized water	
• Blueing in ammonium water	1 min
• Rinse three times in deionized water	
• Tap off excessive water	
• Mount with Kaiser´s glycerol gelatine	

13.5 Double immunohistochemistry for paraffin sections

Step	Time
• Deparaffinize slides through a series of graded alcohols	
• Xylene 1	10 min
• Xylene 2	10 min
• Xylene / 100% Ethanol	5 min
• 100% Ethanol	3 min
• 96% Ethanol	3 min
• 96% Ethanol	3 min
• 70% Ethanol	3 min
• Deionized water	5 min
• Arrange slides in plastic cuvette filled with antigen retrieval solution	
• Heat induced antigen retrieval in a pressure cooker at 120°C	7 min
• Cool down at room temperature	20 min
• Deionized water	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash three times in 1 x TBS-T	2 min, each
• Incubate with Hydrogen Peroxide Block	10 min
• Wash three times in 1 x TBS-T	2 min, each
• Incubate with Ultra V Block	10 min
• Tap off UV-Block and apply primary antibody-cocktail (combine host: rabbit & mouse), incubate at room temperature	30 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply Multivision Polymer Cocktail	30 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply LVBlue (prepare fresh! 2,5 ml AP-buffer + 1 drop LVBlue vial 1 + 1 drop LVBlue vial 2 + 1 drop LVBlue vial 3)	10 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply LVRed (prepare fresh! 5 ml deionized water + 1 drop LVRed vial 1 + 1 drop LVRed vial 2 + 1 drop LVRed vial 3 + 1 drop LVRed vial 4)	10 min
• Wash three times in deionized water	2 min, each
• Dry tissue section at 60°C on a hot plate	60 min
• Mount with xylene-substitute containing medium provided with the kit	

13.6 Double immunofluorescence for cryosections 1	
• Thaw cryosections in glass cuvette	10 min
• Air-dry	10 min
• Fixation in acetone at room temperature	5 min
• Air-dry	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash in 1 x PBS	5 min
• Incubate with Ultra V Block supplemented with 10% human AB-serum	8 min
• Wash three times in 1 x PBS	2 min, each
• Apply primary antibody cocktail (combine host: mouse & rabbit) and incubate at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply secondary antibody cocktail (Alexa Fluor®555 goat anti-mouse IgG and Alexa Fluor®488 goat anti-rabbit IgG) at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Counterstain with DAPI (diluted 1:2000 in 1 x PBS)	10 min
• Wash three times in deionized water	2 min, each
• Mount in ProLong® Gold antifade reagent	
• Store at 4°C	

13.7 Double immunofluorescence for cryosections 2	
• Thaw cryosections in glass cuvette	10 min
• Air-dry	10 min
• Fixation in acetone	5 min
• Air-dry	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash in 1 x PBS	5 min
• Incubate with Ultra V Block supplemented with 10% human AB-serum	8 min
• Wash three times in 1 x PBS	2 min, each
• Apply primary antibody (host: mouse) and incubate at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply secondary antibody (Alexa Fluor®555 goat anti-mouse IgG) at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply second direct fluorescent labeled primary antibody (host: mouse)	30 min
• Wash three times in 1 x PBS	2 min, each
• Counterstain with DAPI (diluted 1:2000 in 1 x PBS)	10 min
• Wash three times in deionized water	2 min, each
• Mount in ProLong® Gold antifade reagent	
• Store at 4°C	

13.8 Triple immunofluorescence for cryosections	
• Thaw cryosections in glass cuvette	10 min
• Air-dry	10 min
• Fixation in acetone	5 min
• Air-dry	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash in 1 x PBS	5 min
• Incubate with Ultra V Block supplemented with 10% human AB-serum	8 min
• Wash three times in 1 x PBS	2 min, each
• Apply primary antibody cocktail (combine host: mouse & rabbit) and incubate at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply secondary antibody cocktail (20µg/ml Alexa Fluor®555 goat anti-mouse IgG and 5µg/ml Cy 5 conjugated AffiniPure goat-anti-rabbit IgG) at room temperature	30 min
• Wash three times in 1 x PBS	2 min, each
• Apply second direct fluorescent labeled primary antibody (host: mouse)	30 min
• Wash three times in deionized water	2 min, each
• Mount in ProLong® Gold antifade reagent	
• Store at 4°C	

13.9 Double immunofluorescence for whole confrontations	
• Perform all steps in 96-well plate	
• Wash confrontations three times in Hanks' BSS	2 min, each
• Fixation in ice cold acetone	30 min
• Wash three times in 1 x TBS-T	5 min, each
• Incubate with primary antibody cocktail (FITC-conjugated Cytokeratin (MNF116) & RPE-conjugated Anti-Human HLA Class I Antigen (W6/32)) at 4°C on a shaking platform	Overnight
• Wash four times in 1 x TBS-T	3 min, each
• Counterstain with ToPro3 (diluted 1:500 in 1 x TBS)	8 min
• Wash three times in 1 x TBS-T	5 min, each
• Prepare slides with reinforcing rings as spacer	
• Mount tissues in the centre of the spacers with ProLong® Gold antifade reagent	
• Store at 4°C	

13.10 Immunofluorescence for thick cryosections	
• Thaw cryosections in glass cuvette	10 min
• Air-dry	10 min
• Fixation in acetone	5 min
• Air-dry	5 min
• Encircle tissue sections with PAP-Pen (optionally)	
• Wash in 1 x TBS-T	5 min
• Incubate with Ultra V Block supplemented with 10% human AB-serum	8 min
• Wash three times in 1 x TBS-T	2 min, each
• Apply primary antibody (host: mouse) and incubate at 4°C	Overnight
• Wash three times in 1 x TBS-T	2 min, each
• Apply secondary antibody (Alexa Fluor®555 goat anti-mouse IgG) at room temperature	30 min
• Wash three times in 1 x TBS-T	2 min, each
• Counterstain with ToPro3 (diluted 1:500 in 1 x TBS)	8 min
• Wash three times in deionized water	2 min, each
• Mount in ProLong® Gold antifade reagent	
• Store at 4°C	

13.11 Denudation of indirect confrontation co-cultures 1

- Prewarm media to 37°C, place placenta in a Petri dish with prewarmed media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2mm pieces, cut villi into pieces of corresponding size (15-20mg)
- Fill a spinner flask with 25ml media
- Preculture of decidua at 37°C, 5% CO_2 on a magnetic stirrer at ~130rpm for 72h
- Change media in spinner flasks after 48h
- Place villi in Petri dish with 20ml media
- Preculture of villi at 37°C, 2.5% O_2 and 5% CO_2 for 72h in a hypoxia workstation (cover Petri dish with aluminium foil if used, hypoxia workstation is translucent)
- Start confrontation co-culture after 72h of preculture as follows
- Transfer decidua pieces and villi separate into small Petri dishes with fresh media
- Fill perforated 2ml reaction tubes with 500µl media and place in a tube rack with lid
- Wash decidua pieces twice in HBSS
- Transfer decidua pieces into Petri dish filled with 10x Tryspin-EDTA (0.5% trypsin, 0.2% EDTA, PAA)
- Transfer control decidua pieces into Petri dish filled with HBSS
- Incubate samples and controls for 3min in an incubator at 37°C, shake gently once between incubation time
- Wash decidua pieces twice in fresh media to stop enzyme activity
- Transfer decidua pieces and villi separate into small Petri dishes with fresh media
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua and villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze at -80°C until sectioning

13.12 Denudation of indirect confrontation co-cultures 2

- Preheat media to 37°C, place placenta in a petridish with preheated media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2 mm pieces, cut villi into pieces of corresponding size (15-20 mg)
- Fill a spinner flask with 25 ml media
- Transfer decidua pieces into spinner flask with shortened pipette tip
- Preculture od decidua for 37°C, 5% CO_2 on a magnetic stirrer at ~130 rpm for 72 hours
- Change media in spinnerflasks after 48 hours
- Place villi in petridish with 20 ml media
- Preculture of villi for 37°C, 2.5% O_2 and 5% CO_2 for 72 hours in a hypoxia workstation (cover petridish with aluminium foil if used hypoxia workstation is translucent)
- Start confrontation co-culture after 72 hours of preculture
- Transfer decidua pieces and villi into small petridishes with fresh media
- Fill perforated 2ml reaction tubes with 500 µl media and place in a tube rack with lid
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua and villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze at -80°C until sectioning

13.13 Oxidative stress in direct confrontation co-cultures 1

- Prewarm media at 37°C, place placenta in a Petri dish with prewarmed media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2mm pieces, cut villi into pieces of corresponding size (15-20mg)
- Transfer decidua pieces and villi into small Petri dishes with fresh media
- Fill perforated 2ml reaction tubes with 500µl media and place in a tube rack with lid
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua and villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze at -80°C until sectioning

13.14 Oxidative stress in direct confrontation co-cultures 2

- Prewarm media to 37°C, place placenta in a Petri dish with prewarmed media
- Select putative decidua parietalis and villi from the tissue sample
- Embed corresponding tissue samples in cryosectioning compound for control
- Cut decidua into 1-2mm pieces, cut villi into pieces of corresponding size (15-20mg)
- Wash ready dissected tissues in fresh media
- Transfer decidua pieces and villi into small Petri dishes with culture media supplemented with 0, 2 or 4mM H_2O_2
- Wash ready dissected tissues in fresh media
- Fill perforated 2ml reaction tubes with 500µl media and place in a tube rack with lid
- Transfer one decidua piece and one piece of villi into each reaction tube
- Control tubes are filled with decidua and villi alone, respectively
- Embed corresponding tissue samples in cryosectioning compound for control
- Close lid of tubes and of tube rack and place in incubator at 37°C, 5% CO_2 for 72h
- After confrontation co-culture check tissues for adhesion
- Embed confrontations carefully in cryosectioning compound in the appropriate orientation under the dissecting microscope
- Freeze tissues at -80°C until sectioning
- Centrifuge supernatant at 10,000rcf for6 min
- Transfer supernatant into fresh tube and store at -80°C until LDH-measurement