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ORIGINAL ARTICLE *Early pregnancy*

Extravillous trophoblast invasion of venous as well as lymphatic vessels is altered in idiopathic, recurrent, spontaneous abortions

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STUDY QUESTION: Do extravillous trophoblasts (EVTs) invade non-arterial decidual vessels in healthy and pathological pregnancies?

SUMMARY ANSWER: Our results reveal that trophoblast invasion of venous and lymphatic vessels is a frequent event during the first trimester of pregnancy and is compromised in recurrent spontaneous abortion (RSA). In addition, the present data suggest that EVTs populate regional lymph nodes during pregnancy.

WHAT IS ALREADY KNOWN: Human trophoblasts remodel and invade decidual spiral arteries. In addition, a recent report demonstrates that trophoblasts contact and invade decidual veins.

STUDY DESIGN, SIZE, DURATION: Tissue samples of human first trimester deciduae basalis ($n = 54$, 6th–13th weeks of gestation) obtained from elective pregnancy terminations were used to study trophoblast invasion into veins and lymphatics, in comparison to arteries. Age-matched cases of idiopathic, recurrent spontaneous abortions tissue samples ($n = 23$) were assessed for cell numbers of EVTs in these decidual vessels. In addition, lymph nodes of four pregnant women were analysed for the presence of EVTs.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Localization, frequency and EVT-mediated targeting and invasion of arterial, venous as well as lymphatic vessels were determined in first trimester decidua basalis tissue sections using immunofluorescence staining with antibodies against CD31, CD34, ephrin B2 (EFNB2), ephrin receptor B4 (EPHB4), HLA-G, podoplanin, prospero-related homeobox 1 (Prox-1), alpha-smooth muscle actin 2 (ATCTA2), von willebrand factor (vWF) and proteoglycan 2 (PRG2). Arterial, venous and lymphatic-associated EVTs were further characterized according to their position in the vascular structure and classified as intramural (im) or intraluminal (il).

MAIN RESULTS AND THE ROLE OF CHANCE: EVTs, specifically expressing PRG2, target and invade veins and lymphatics in first trimester decidua basalis since HLA-G+ trophoblast were detected in the vascular wall (intramural EVT, imEVTs) and in the lumen of these vessels (intraluminal EVT, ilEVTs). In total, 276 arteries, 793 veins and 113 lymphatics were analysed. While EVTs contact and invade arteries and veins to a similar extent we found that lymphatics are significantly less affected by EVTs ($P = 0.001$). Moreover, ilEVTs were detected in the lumen of venous and lymphatic vessels, whereas ilEVTs were only found occasionally in the lumen of arteries. Interestingly, RSA tissue sections contained significantly more arterial ($P = 0.037$), venous ($P = 0.002$) and lymphatic vessels ($P < 0.001$), compared to healthy controls. However, while RSA-associated arterial remodeling was unchanged ($P = 0.39$) the ratios of EVT-affected versus total number of veins

($P = 0.039$) and lymphatics ($P < 0.001$) were significantly lower in RSA compared to age-matched healthy decidual sections. Finally, HLA-G+/PRG2+/CD45-EVTs can be detected in regional lymph nodes of pregnant women diagnosed with cervical cancer.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: In this study, first trimester decidual tissues from elective terminations of pregnancies have been examined and used as a reference for healthy pregnancy. However, this collective may also include pregnancies which would have developed placental disorders later in gestation. Due to limitations in tissue availability our staining results for EVT-specific marker expression in regional lymph nodes of pregnant women are based on four cases only.

WIDER IMPLICATIONS OF THE FINDINGS: In this study, we propose migration of HLA-G^+ cells into regional lymph nodes during pregnancy suggesting that the human EVT is capable of infiltrating maternal tissues via the blood stream. Moreover, the description of compromised EVT invasion into the venous and lymphatic vasculature in RSA may help to better understand the pathological characteristics of idiopathic recurrent pregnancy loss.

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Key words: trophoblast / decidual vasculature / vascular remodeling / extravillous trophoblast / recurrent spontaneous abortion

Introduction

Almost a century ago, Grosser (1927) was the first to suggest that cells of trophoblastic origin are found in uterine arteries at mid gestation. These trophoblasts were referred to as extravillous trophoblasts (EVTs) as they detach from placental villi and invade the decidua basalis. Later on, it has been postulated that EVTs are central to pregnancy-specific remodeling of spiral arteries by associating the presence of intravascular trophoblast with the appearance of fibrinoid and degenerative changes within in the vascular wall of decidual and myometrial arteries (Hamilton and Boyd, 1966; Pijnenborg *et al.*, 1980). Importantly, trophoblast-mediated remodeling provokes conversion of spiral arteries with narrow lumina into larger conduits delivering low-pressure, high blood flow to the growing fetus. These arterial changes were found to be vastly absent in hypertensive pregnancies and intrauterine growth restriction suggesting trophoblastic malfunction (Brosens *et al.*, 1972; Khong *et al.*, 1986a,b). The rapid development of immunohistochemical (IHC) and fluorescence-based techniques then provided definitive evidence for the involvement of trophoblasts in pregnancy-associated spiral artery remodeling (Bulmer *et al.*, 1986; Khong *et al.*, 1986a,b). These afore-mentioned groundbreaking studies and many others have set the basis for our current understanding of how trophoblasts are involved in the adaptation of the uterine arterial vasculature during pregnancy (reviewed in (Pijnenborg *et al.*, 2006)). More recently, advanced molecular studies have shown that trophoblasts are capable of inducing apoptosis in smooth muscle as well as endothelial cells (Red-Horse *et al.*, 2006; Keogh *et al.*, 2007) and secrete certain proteases such as MMP-12 inducing elastolysis of the vascular wall (Harris *et al.*, 2010). In addition, it has been suggested that initial steps of arterial remodeling are accomplished by certain decidual immune cell populations including macrophages and uterine natural killer cells (Smith *et al.*, 2009).

Apart from their appearance in the vascular wall and lumen of spiral arteries, early studies on hysterectomies and legal abortions in the 1960s also suggested that trophoblasts contact, invade and erode decidual glandular epithelium (Hamilton and Boyd, 1960). In particular, this phenomenon was found to occur in early stages of placental development. Recently, EVT-mediated invasion into glandular epithelium was confirmed by using antibody-based IHC analyses (Moser *et al.*, 2010, 2015). This process may facilitate histiotrophic nutrition of the growing embryo as it has been shown that endometrial glands discharge into the intervillous space (Burton *et al.*, 2002). Although interaction between trophoblasts and uterine veins in macaques and humans has already been proposed in the 1990s and early 2000s (Blankenship *et al.*, 1993; Craven *et al.*, 2000) no further studies on this phenomenon have been reported until recently (see below). One reason for the lack of continuing investigations may be due to a scientific report suggesting that EVT do not contact and invade decidual veins because ephrin B1+ and ephrin B2+ EVTs do not adhere to ephrin receptor B4 (EPHB4)-coated substrates *in vitro* (Red-Horse *et al.*, 2005). However, this assumption has recently been challenged by demonstrating that EVT-mediated targeting and invasion into EPHB4-positive decidual veins occur during the first trimester of human pregnancy (Moser *et al.*, 2016). In contrast, our knowledge about a functional lymphatic system and related trophoblast function in the decidua remains controversial and poorly defined. While two scientific papers reported lymphangiogenesis during decidual development (Red-Horse *et al.*, 2006) others propose the opposite by suggesting that endometrial lymph vessels regress during pregnancy (Volchek *et al.*, 2010). In addition, it has been shown that outgrowing EVTs from transplanted human placental villi induce lymphangiogenesis in severely compromised immunodeficient mice. The authors also describe that EVTs localize in close vicinity to LYVE-1+ lymphatic endothelial cells (LECs) (Red-Horse *et al.*, 2006). However, whether or not trophoblasts contact and/or invade lymphatic vessels in the decidua remains elusive.

Failures in trophoblast-mediated remodeling of spiral arteries have repeatedly been associated with adverse pregnancy outcome. However, little is known about possible interactions between invading EVTs and venous as well as lymphatic vessels. Therefore, the scope of this study was to thoroughly investigate whether human trophoblasts target and/or invade non-arterial vessels during the first trimester of pregnancy. Our data clearly indicate that EVTs are found in close vicinity to veins as well as lymphatics and actively invade both endothelia.

Strikingly, we also provide evidence that EVTs accumulate in regional lymph nodes of pregnant women diagnosed with cervical cancer. Finally, we found that idiopathic, recurrent spontaneous abortions (RSA) are associated with significantly lower ratios of EVT-affected versus total number of non-invaded venous and lymphatic vessels.

Materials and Methods

Tissue collection

Human first trimester placental ($n = 6$) and decidua basalis ($n = 54$) tissues were obtained from elective pregnancy terminations performed between 6th and 12th weeks of gestation. The gestational age was determined by ultrasound and vacuum suction was done after patients were locally anaesthetized. Age-matched paraffin-embedded decidua basalis tissues of RSA cases ($n = 23$) were received from the archive of the Clinical Institute of Pathology, Medical University of Vienna. Therefore, a computer-based search was conducted to identify women with the diagnosis of idiopathic RSA between the years 2006–2016 and whose abortive material was histologically examined at our Clinical Institute. The presence of decidua basalis on these samples was confirmed by an independent pathologist.

Similarly, paraffin-embedded lymph node specimens ($n = 4$) of pregnant women were obtained retrospectively from the archives of the Clinical Department of Pathology from both the Medical University of Vienna and Bonn. These lymphadenectomies were performed due to diagnoses of maternal malignancies during pregnancy. Final examination and tumor staging were performed by an independent pathologist. Tumor characteristics are found in Table I. All tissues were collected with written informed consent and utilization was approved by the Ethics Committee of the Medical University of Vienna and Bonn.

Patient samples

Diagnosis of idiopathic RSA was defined as miscarriages of three or more consecutive pregnancies before 20 weeks of gestation of unexplained origin and based on ultrasound examination. Women were excluded if one of the following screening investigations revealed a possible contributing factor for their pregnancy losses: antiphospholipid syndrome, thrombophilia (activated protein C resistance, Leiden factor V mutation, prothrombin gene mutation, protein C and S deficiency, antithrombin III deficiency), uterine anomaly, hormonal dysfunction (polycystic ovarian syndrome, diabetes mellitus, abnormal thyroid function, hyperprolactinemia, luteal insufficiency), folate deficiency, infections (chlamydia, toxoplasmosis and ureoplasma) or chromosomal aberrations. Baseline characteristics of the included patients ($n = 23$) are shown in Table II. Moreover, patients enrolled for lymph nodal evaluation ($n = 4$) were diagnosed with either breast ($n = 2$) or cervical ($n = 2$) cancer during pregnancy and, therefore, lymphadenectomies were performed (Table I).

Immunofluorescence stainings of paraffin-embedded tissue

First trimester placental and decidual tissues were fixed with 7.5% (w/v) formaldehyde and embedded in paraffin (Merck, Darmstadt, Germany). Serial sections (3μm) were cut using a microtome (HM355; Microm), deparaffinized and antigens were retrieved in PT Module Buffer 1 (100× citrate buffer, pH 6; Thermo Fisher Scientific) or PT Module Buffer 4 (100× Tris-EDTA, pH9) by KOS MicrowaveStation (Milestone Srl, Sorisole, Italy). Subsequently, sections were blocked in Blocking Buffer (1 × PBS/5%fish skin/0.3%TritonTM X-100) and incubated with primary antibodies listed in Supplementary data, Table SI overnight at 4°C. Afterwards, slides were washed three times and incubated for 1 h with secondary antibodies (2 μg/ml) (Supplementary data, Table SI). Nuclei were stained with 1 μg/ml DAPI. Images were acquired on a fluorescence microscope (Olympus BX50) and digitally photographed at ×200 and ×400 magnifications (CC12 digital camera, CellˆP software, Olympus, Hamburg, Germany). To make immunofluorescence (IF) double stainings visible for color-blind readers, red images were replaced by magenta (Hex Code: #FF00FF). All IF stainings were analyzed by two independent investigators.

Identification of decidual vessels and quantification of EVT invasion

All decidual tissues were stained with haematoxylin and eosin (H&E) as well as for HLA-G to assess morphology and type of decidua. Based on these analyses, tissues were classified as decidua basalis when prominent invasion by HLA-G+EVTs was noticed. Selected decidua basalis tissues were further analyzed by CD31, CD34, von willebrand factor (vWF) and HLA-G stainings to select for regions with comparable vascular densities and EVT invasion. In addition, samples were stained with H&E and keratin (KRT) to visualize decidual glands. Finally, three sections per tissue showing comparable densities of vessels, glandular structures as well as EVTs were

Table I Tumor characteristics.

Case	Diagnosis	FIGO stage	Histopathology					Therapy	
			Lymph node metastasis	LVI	BVI	Grade	Resection margins	Neoadjuvant CHT during pregnancy	Surgical treatment (GA, wks)
1	N. mammae dext., Local recurrence	4D	Present	Present	Present	3	1	Absent	Surgical treatment of the local recurrence and CD (33)
2	N. mammae sin.	3	Absent	Absent	Absent	3	0	Absent	Total mastectomy with axillary lymphadenectomy and CD (37)
3	N. cervicis	1B1	Absent	present	Absent	3	0	4 cycles Cisplatin	CD and radical hysterectomy with pelvic lymphadenectomy (25)
4	N. cervicis	1B1	Absent	Present	Absent	2	0	Absent	Radical hysterectomy with fetus in situ and pelvic lymphadenectomy (18)

CD, cesarean section; LVI, lymphovascular invasion; BVI, blood vessel invasion; CHT, chemotherapy; GA, gestational age; R0, complete resection with microscopically negative margins; R1, resection with cancerous cells that can be seen microscopically; wks, weeks.

Table II Clinical characteristics of the study subjects with RSA ($n = 23$).

	RSA ($n = 23$)
Age	35 ± 1.1 (28–44)
Gravida	5.8 ± 0.74 (3–15)
Para	0.65 (0–2)
GA (wks)	8.5 ± 0.37 (6–12)
BMI	22.7 ± 0.60 (20–26)
Nicotine	9% ± 0.06
ART	0%

Values are given as means ± SEM, with range in parentheses or as percentages. RSA, recurrent spontaneous abortion; GA (wks), gestational age (weeks).

analyzed for arterial, venous and lymphatic vascular density and EVT-mediated invasion into decidual vessels. Identification of arteries, veins and lymphatics was based on morphology and antibody-based IF stainings. Arteries were defined by a thick-walled ACTA2-positive smooth muscle layer and endothelial cells expressing ephrin B2 (EFNB2). Moreover, venous endothelium was characterized by positive EPHB4 staining and lack of EFNB2 expression. Lymphatic vessels were defined by expression of the lymph-associated markers PDPN and Prox-1 (Supplementary Data, Fig 1). In total, 1620 images were analyzed quantitatively. Lymph nodes ($n = 4$) and primary tumor tissues ($n = 2$) were analyzed for the presence of HLA-G+/PRG2+ EVTs by evaluating 12 tissue sections per sample. To ensure objectivity, the quantification of all IF stainings was analyzed by two independent investigators.

Isolation and cultivation of purified primary villous cytotrophoblasts and cell column trophoblasts

Primary villous cytotrophoblasts (vCTBs) and cell column trophoblasts (CCTs) were isolated from pooled first trimester placental tissues ($n = 6$) by two consecutive digestion steps followed by Percoll density gradient centrifugation as recently described in (Haider *et al.*, 2016). Briefly, placental villi were scraped into small pieces (1–3 mm). For isolation of CCTs, the first digestion was performed with 0.125% trypsin (Gibco®, Life Technologies) and 12.5 mg/ml DNase I (Sigma-Aldrich) for 30 min at 37°C. Subsequently, digestion was stopped using 10% (v/v) fetal bovine serum (PAA Laboratories) and cells were filtered through a 100-µm cell strainer (BDBiosciences). To isolate vCTB cells, a second digestion step of the remaining tissue was performed with 0.25% trypsin and 12.5 mg/ml DNase I for 30 min at 37°C and processed as described above. Digestion solutions containing either CCTS or vCTBs were each separated by using Percoll density gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden). Cells that had been collected from the 35 to 50% Percoll layer were incubated with red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 5 min at room temperature to remove containing red blood cells and subsequently washed with 1 × HBSS. Cells were then plated (45 min) in culture medium (DMEM/Ham's F-12, 10% (vol/vol) fetal calf serum, 0.05 mg/ml gentamicin, 0.5 µg/ml fungizone; Gibco) allowing for adherence of contaminating stromal cells. Finally, nonadherent trophoblasts were collected and CCTs and vCTBs were seeded onto fibronectin-coated dishes at a density of 2.5×10^5 cells per square centimeter and 3.25×10^5 cells per square centimeter, respectively. CCT and vCTB cells were harvested after 96 h. Cells were snap frozen for western blotting.

Western blotting

Cell lysates were prepared according to standard protocols as recently described (Sonderegger *et al.*, 2010). Protein extracts were separated by SDS-PAGE, blotted onto methanol-activated polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK) and incubated overnight at 4°C with primary antibodies as listed in Supplementary data, Table SI. Finally, blots were incubated for 1 h at room temperature with horse-radish peroxidase (HRP)-conjugated secondary antibodies (Supplementary data, Table SI). Signals were developed using ECL Prime Detection Kit (GE Healthcare) and visualized with FluorChemQ Imaging System (Alpha Innotech).

Statistical analysis

According to sample size calculation, we analyzed ≥46 tissue samples for IF analyses to obtain a power of at least 0.95 with a two-sided Type I error of 0.05. Data are presented as mean ± SEM and were analysed using IBM SPSS Statistics 23.0 software. Before analysis, data were log-transformed because of their skewed distribution. Gaussian distribution and equality of variances were examined with Kolmogorov–Smirnov, Shapiro–Wilk and Levene's tests. Comparisons of more than two groups (veins, arteries and lymphatics) were performed by using repeated measures ANOVA, whereas for direct comparisons (healthy versus RSA pregnancies) unpaired Student's *t*-tests were used. Bonferroni corrections were used as *post hoc* and multiple comparison tests. A *P*-value of <0.05 was considered statistically significant.

Results

EVT target and invade decidual venous and lymphatic vessels

To determine whether human invasive trophoblasts also invade non-arterial vessels during the first trimester of pregnancy IF staining of first trimester decidual tissues was performed. To this end, we first established antibody staining in order to discriminate between different types of vessels (Supplementary data, Fig 1). These data revealed the presence of numerous venous and lymphatic vessels in decidua basalis tissue sections (Fig. 1D and Supplementary data, Fig 1). Artery-associated trophoblasts were defined according to their localization in the arterial smooth muscle layer and/or by their close contact to an intact endothelial cell lining and referred to as intramural EVTs (imEVTs). The latter criterion was also applied to define trophoblast-associated attachment to venous and lymphatic vasculature. In addition, we detected EVTs that were either integrated in the vascular endothelium or found inside the lumen of decidual vessels. These cells were defined as intraluminal EVTs (ilEVTs) (Fig. 1 A–C). First, we found that veins are the most abundant vessel type in decidua basalis followed by arteries and lymphatics, respectively. Hence, to account for these differences in absolute numbers we determined the ratio between EVT-affected and the total number for each type of vessel. While EVTs contact and invade arteries and veins to a similar extent we found that lymphatics are significantly less affected by EVTs (Fig. 1D). Moreover, we found that arteries are generally associated with a higher number of imEVTs when compared with veins and lymphatics. On the other hand, both veins and

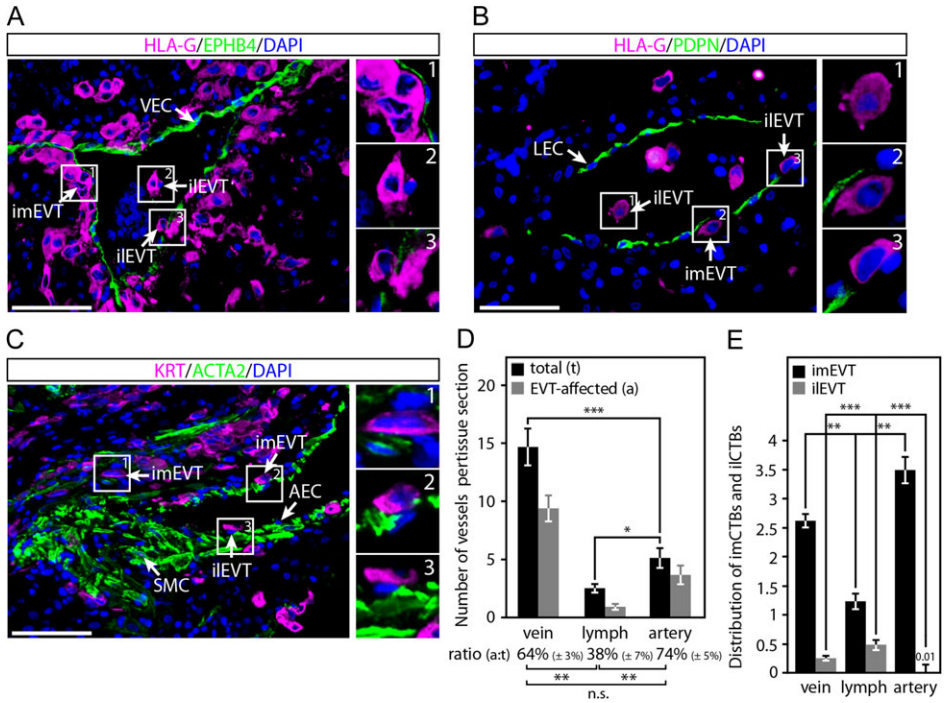

Figure 1 EVTs contact and invade decidual veins and lymphatics (**A–C**) IF co-stainings of HLA-G and EPHB4 (A), HLA-G and PDPN (B), and KRT and ACTA2 (C) were performed on decidual tissue sections (9th week). Digitally zoomed insets display localization of imEVTs and ilEVTs as indicated. Scale bars: 50 μm. The bar graph in (**D**) represents the mean number (±SEM) of total (black bars) and EVT-affected (gray bars) veins, lymphs and arteries per tissue sections ($n = 54$, mean area, 0.5 cm^2; ± 0.25). In the bottom, the ratio between EVT-affected and the total number of vessels is shown (%, mean number per tissue section, ± SEM). In (**E**) mean numbers (±SEM) of imEVTs and ilEVTs per vessel are shown. DAPI (blue) was used to visualize cell nuclei. Data were quantified from 10 randomized fields per tissue section. three sections per sample ($n = 54$) were analysed. Statistical significance was determined by using repeated measures ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s., not significant. a, EVT-affected; AEC, arterial endothelial cell; LEC, lymphatic endothelial cell; SMC, smooth muscle cell; t, total; VEC, venous endothelial cell; EPHB4, ephrin receptor B4; imEVTs, intramural extravillous trophoblast; ilEVTs, intraluminal extravillous trophoblast; IF, immunofluorescence; EVTs, extravillous trophoblasts; KRT, keratin.

lymphatics contained significantly more ilEVTs than arterial vessels (Fig. 1E). Taken together, these results indicate that EVTs also contact and invade venous as well as lymphatic vessels as these cells are found in the vascular wall and in the lumen of the non-arterial decidual vasculature.

PRG2 is a specific marker for EVTs in the fetal-maternal interface

Since our analyses indicate that ilEVTs are also found in the lumen of venous and lymphatic vessels, we postulated that these cells might accumulate in uterine lymph nodes during pregnancy. To test this hypothesis, we examined lymph node biopsies from pregnancies affected by cervical cancer. First, to screen for lymph node-infiltrating trophoblasts we decided to use, beside of HLA-G, an additional marker meeting the following criteria: placenta specific, enriched in EVTs and absent from unaffected lymph nodes as well as cervical cancer cells. To this end, we searched the Human Protein Atlas (HPA) for placenta-enriched factors (Supplementary data, Fig. 2A) and screened for their mRNA expression levels in EVTs compared to non-invasive villous trophoblasts (Supplementary data, Fig. 2B). Of note, HLA-G was not annotated as placenta-enriched in the HPA since its mRNA seems to be more abundantly expressed (data not shown). In a next step, we stained placental villi and decidua basalis tissue sections for the expression of three selected, putative EVT markers including proteoglycan 2 (PRG2, also known as eosinophil granule major basic protein), chorionic somatomammotropin 1 (CSH1, also known as placental lactogen 1, PL) and pregnancy-associated plasma protein-A2 (PAPPA2). Further analyses including double IF stainings with KRT7 revealed that only PRG2 is specific to EVTs whereas the others are present in both EVTs and CTBs but are absent from all other trophoblast subtypes (Fig. 2A and Supplementary data, Fig. 3A). Western blot analysis of *in vitro* differentiated CTBs and EVTs confirmed specific expression of PRG2 in the latter population (Fig. 2B). Interestingly, we could also detect high levels of PRG2 in imEVTs as well as ilEVTs of both venous and lymphatic vessels (Fig. 2C and D). Importantly, IHC staining of PRG2 and HLA-G in the Human Cancer Protein Atlas was annotated as 'absent' in cervical and all other cancer tissues tested (Supplementary data, Fig. 3B), whereas IHC stainings in normal

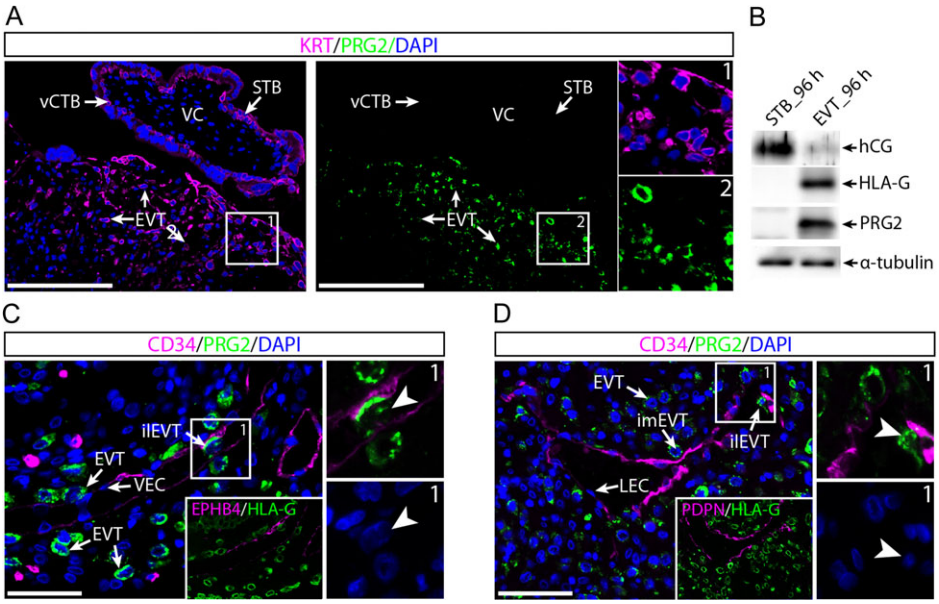

Figure 2 PRG2 is expressed in EVTs that contact and invade venous and lymphatic vessels. (**A**) IF co-staining of KRT and PRG2 in a first trimester decidual tissue section (10th week). Digitally zoomed insets display co-expression of KRT (1) and PRG2 (2). Scale bars: 100 μm. (**B**) Western blot analysis of PRG2 in primary differentiated STBs and EVTs (6th–12th weeks). hCG and HLA-G expression was determined to control for STB and EVT differentiation. α-Tubulin served as loading control. (**C**, **D**) IF co-staining for CD34 and PRG2 were performed on first trimester placental tissue (9th week). Digitally zoomed insets display ilEVTs (arrow heads) in venous (C) and lymphatic (D) vessels. Insets show immunofluorescent co-stainings for EPHB4 (C) or PDPN (D) and HLA-G of serial sections. DAPI (blue) was used to visualize cell nuclei. Scale bars: 50 μm. STB, syncytiotrophoblast; vCTB, villous cytotrophoblast; VC, villous core.

tissues showed the expected expression pattern for both markers (Supplementary data, Fig. 3 A).

EVTs accumulate in uterine lymph nodes of pregnant women with cervical cancer

Based on our prior data, we used PRG2 and HLA-G as markers for putative, infiltrating EVTs into lymph nodes resected from two women affected by cervical cancer. Importantly, both cases were reported to show no tumor cell invasion into regional lymph nodes. First, we performed co-stainings with HLA-G and CD45. Interestingly, we were able to detect HLA-G+/CD45- cells in both samples, demonstrating the presence of trophoblastic features in cells of non-leukocyte origin (Fig. 3A). Moreover, we found HLA-G+ cells in the lumen of MECA-79+ vessels, suggesting that trophoblasts may infiltrate uterine lymph nodes via so-called high endothelial venules (HEVs) (Fig. 3B). Subsequent co-stainings with HLA-G and PRG2 revealed the presence of cells with exclusive marker gene expression pattern of EVTs again in both cases analysed (Fig. 3 C). Corresponding primary tumor tissue samples were negative for both markers tested (Supplementary data, Fig. 3C). The frequency of HLA-G/PRG2 double positive cells in regional lymph nodes was below of 1% of total cells per visual field. In parallel, we also stained resected peripheral axillary lymph nodes from pregnant women diagnosed with breast cancer (Table I) as controls. Both cases investigated, showed no positive IF stainings for neither HLA-G nor PRG2 (data not shown). These data suggest that ilEVTs may infiltrate regional lymph nodes during pregnancy.

Trophoblast-associated invasion into veins and lymphatics is compromised in RSAs

Since RSA cases have already been connected with altered trophoblast invasion we were interested whether EVT-mediated invasion into the decidual vasculature is changed in these tissues. To this end, we evaluated 23 cases of RSA and 23 healthy age-matched controls. Firstly, we found that RSA tissues generally contained significantly more vessels than healthy control decidual sections (Fig. 4A). In a next step, we wondered whether the ratio of vessels that are associated with EVTs within their lumen or vascular wall and the total number of vessels would differ between RSA and healthy controls. Interestingly, decidua basalis RSA tissues show a significantly lower ratio of affected veins and lymphatics versus the total number of vessels, respectively when compared to age-matched healthy controls. We therefore conclude that EVT-associated invasion into non-arterial vessels including veins and lymphatics is compromised in RSA.

Discussion

To date, numerous papers have been published to characterize and functionally assess EVT-mediated spiral artery remodeling in healthy as well as complicated pregnancies. In addition, it is now well-accepted that EVTs also invade and erode decidual glands perhaps to establish histotrophic nourishment during early pregnancy (Burton *et al.*, 2002). However, whether or not invasive trophoblasts target decidual veins and/or lymphatics is far from clear as very little data exist to answer

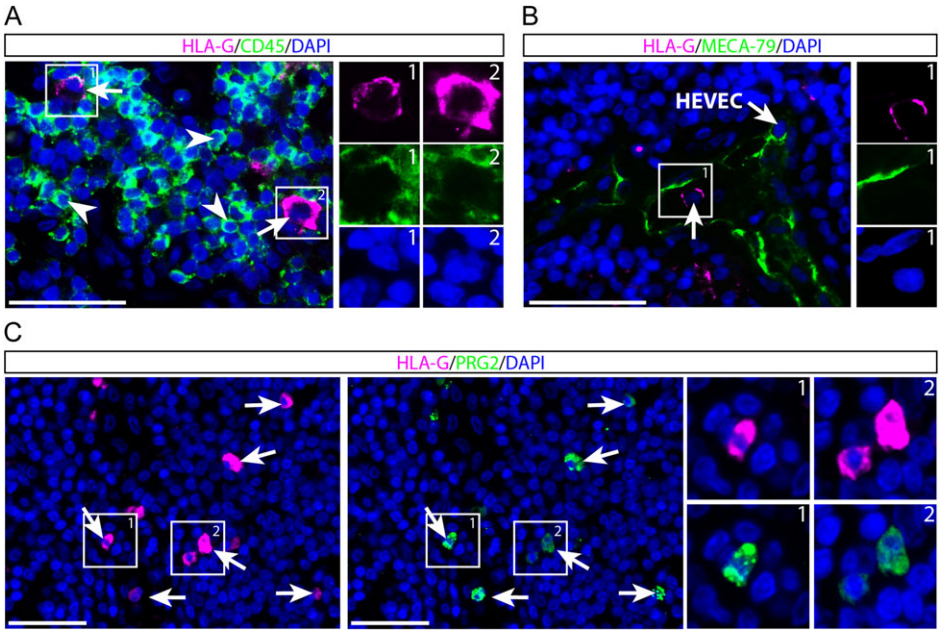

Figure 3 Cells with characteristics of EVTs accumulate in regional lymph nodes of pregnant women. (**A–C**) IF co-staining for HLA-G and CD45 (A), HLA-G and MECA-79 (B), HLA-G and PRG2 (C) were carried out on resected lymph node tissue (18th week). (A) Digitally zoomed insets display HLA-G+/CD45- cells (A), HLA-G+ cells in the lumen of MECA-79+ HEVs (B) and HLA-G+/PRG2+ (C) cells in lymph node tissue. Arrows depict HLA-G+/CD45- and HLA-G+/PRG2+ cells, respectively. Arrow heads mark HLA-G-/CD45+ cells in (A). DAPI (blue) was used to visualize cell nuclei. Representative pictures of 10 randomized fields per tissue section are shown. 10 sections per sample ($n = 2$) were analysed. Scale bars: 50 μm. HEVEC, high endothelial venule endothelial cell.

this question. One obvious problem to be addressed is that remodeled arteries look in some respect more like veins as they appear thin-walled and convoluted (Pijnenborg *et al.*, 1983). In addition, access to early decidual material when arterial invasion by EVTs is in its initial phase and thus arteries and veins would be more easily distinguishable from each other, is greatly limited. As already mentioned above, Moser *et al.* (2016) have recently proposed that EVTs indeed invade into venous vessels in the first trimester of pregnancy. To prove this, they took advantage of specific marker expression and typical morphological appearance including EPHB4-positive endothelium, the absence of a tunica media and the presence of desmin. By screening a larger cohort of decidua basalis tissue sections we here confirm these findings. Moreover, Moser *et al.* compared the absolute numbers of EVT-affected arteries and veins and conclude that the latter are more often targeted and invaded by trophoblasts. We agree that in total more veins are affected by EVTs as arteries since in general venous vessels are more abundant in the decidua than arteries. However, the ratio between the total number of respective vessels and those that are affected by EVTs showed no difference when comparing arteries and veins. By more in depth analysis, we however found that arteries are generally associated with a higher number of imEVTs and venous vessels contain significantly more ilEVTs than arterial vessels.

In a next step, we stained decidual tissue sections for the presence of lymphatics. Indeed, we were able to find vessels with strong positivity for the well-accepted LECs markers PDPN and Prox-1. Although, the number of lymphatics detected and the ratio between the total and EVT-affected vessels were significantly decreased when compared to arterial or venous vessels our results contrast a recent report proposing that lymphatic vessels are virtually absent in the decidua (Volchek *et al.*, 2010). Given that the first trimester of pregnancy is accompanied by massive tissue erosion of glands and stroma the presence of a functional lymphatic system does not appear surprising. In this context, it is interesting to note that we found the highest number of ilEVTs associated with lymph vessels suggesting that trophoblasts may induce lymphatic drainage of extracellular fluid. Along those lines, although a continuous blood flow between mother and embryo does not occur before the 10th–12th week of pregnancy, arterial blood has been noticed to accumulate in the decidua and trophoblastic lacunae and it has been suggested that it leaves via the venous blood system (Hamilton and Boyd, 1960). This again implies that EVT-mediated invasion into non-arterial vessels may facilitate drainage of body fluids. In addition, veins and lymphatics are an essential part of the immune system, the former enabling immune cell migration into tissues, the latter facilitating immune cell trafficking to lymph nodes. In this context, it is tempting to speculate that EVTs, which are associated with non-arterial vessels, might play a regulatory function in immune cell influx and efflux.

In mouse and rat so-called trophoblast giant cells (TGs), the functional equivalent to human EVTs, secrete hormones and cytokines into the circulation ensuring maternal adaptation to pregnancy (Soares *et al.*, 1996; Hu and Cross, 2010). Interestingly, we found strong expression of PRG2 in imEVTs and ilEVTs, associated with veins and

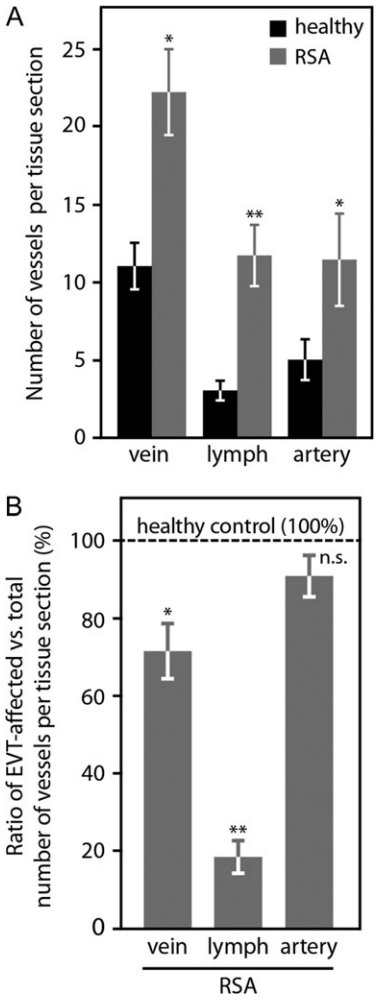

Figure 4 The total number of arterial and non-arterial vessels is increased in RSA tissues, whereas the ratio of EVT-affected versus total number of non-arterial vessels is decreased. (**A**) Bar graphs show the mean number (±SEM) of total veins, lymphs and arteries in healthy (black bars; $N = 23$) versus age-matched RSA tissue sections (gray bars; $N = 23$). (**B**) Bars represent the ratio of EVT-affected versus total number of veins, lymphs and arteries in RSA and aged-matched healthy controls (set to 100%, indicated by a dotted line) per tissue sections (±SEM). Data were quantified from 10 randomized fields per tissue section. three sections per sample ($n = 46$) were analysed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; n.s., not significant. RSA, recurrent spontaneous abortion.

lymphatics. PRG2 expression has already been associated with EVTs, at that time referred to as *x*-cells, and shown to accumulate in the serum of pregnant women (Wasmoen *et al.*, 1987; Oxvig *et al.*, 1995). By demonstrating its absence in villous trophoblast subtypes and maternal decidual cells we propose PRG2 as an exclusive marker for invasive EVTs. We further determined that PRG2 mRNA and protein expression is highly placenta-enriched by screening RNAseq data and antibody staining images available as a public resource from the HPA portal (www.proteinatlas.org). Therefore, we propose EVTs as the likely source for the strong increase in PRG2 serum levels during pregnancy since eosinophils, the only other cell type, know to express PRG2, are absent from the fetal-maternal interface and pregnancy serum (Wasmoen *et al.*, 1987). Along these lines, other eosinophil-derived factors are undetectable in the serum of pregnant women and placental fluids and tissues contain up to 6-fold increased PRG2 concentrations in comparison to peripheral blood (Wasmoen at al., 1987). In pregnancy serum, the pro-form of PRG2 is bound in a complex to PAPPA, in which the latter is inhibited (Overgaard *et al.*, 2004; Sivanandam *et al.*, 2004). The cleaved active form seems to be exclusively produced in eosinophilic granules, where it functions as a potent toxin for helminths, protozoa, bacteria and some mammalian cells. Therefore, EVT-derived PRG2 is likely to function as a regulatory factor of PAPPA-controlled proteolysis. Whether or not EVTs also generate the active form PRG2 is a current focus in our laboratory. Consequently, we propose that the venous and lymphatic decidual system might serve as a secretory route for EVT-derived factors and suggest PRG2 as an interesting marker for the early detection of EVT-associated pregnancy complications.

Furthermore, we examined resected lymph nodes of four pregnant women diagnosed with cervical cancer or breast cancer for the expression of EVT-specific signatures. Indeed, we were able to find cells displaying a CD45-/HLA-G+/PRG2+ phenotype in uterine lymph nodes whereas axillary lymph nodes did not show positive stainings for HLA-G and PRG2. Given that we frequently found EVTs in the lumen of both venous and lymphatic vessels it seems plausible that these cells accumulate in regional lymph nodes. Along those lines, we detected HLA-G+ cells in the lumen of MECA-79+ HEVs, which are specialized veins enabling leukocytes trafficking into lymphoid tissues (Girard *et al.*, 2012). In this context, it is interesting to note that EVTs were shown to express L-selectin, a leukocyte-specific cell surface protein enabling lymphocyte migration into lymph nodes (Genbacev *et al.*, 2003). It has further been suggested that immunomodulatory cancer cell-derived factors including transforming growth factor beta (TGFβ), interleukin-6 and −10 (IL-10) and a variety of chemokines are capable of inhibiting the generation of tumor-specific cytotoxic T cells in sentinel lymph nodes (Cochran *et al.*, 2006). Interestingly, EVTs produce a variety of immune-modulatory factors such as CXCL12 (Hanna *et al.*, 2003), IL-10 (Roth *et al.*, 1996), PDL1 (Petroff *et al.*, 2002; Holets *et al.*, 2006) as well as TGFβ-2 (Tarrade *et al.*, 2001) and thus may exert similar effects on immune cell populations in uterine lymph nodes. Whether, extravasation of EVTs into the vascular lumen and appearance in regional lymph nodes is a consequence of an active or passive process certainly requires further investigation. Given that both veins and lymphatics drain body fluids, escape of EVTs into the blood or lymph system may at least partly occur in a passive manner. On the other hand, a functional venous system requires an intact endothelial cell layer suggesting that cellular efflux underlies an active, invasive process involving erosion of the vascular wall.

Lastly, we screened 23 cases of RSA and age-matched healthy controls for abnormalities in EVT-dependent vascular invasion. Surprisingly, we detected an increased number in arterial, venous and lymphatic vessels in RSA tissues contrasting a previous publication reporting no changes in vascular density of RSA decidua basalis tissues (Vailhe *et al.*, 1999). On the other hand, another scientific study reported that the non-pregnant endometrium of women, diagnosed with RSA, shows an increase in angiogenesis and vessels density (Quenby *et al.*, 2009). Interestingly, we found a significant

RSA-associated decrease in the ratio of affected versus total number of veins and lymphatics when compared to age-matched healthy controls. This phenomenon was particularly pronounced in lymphatics. It may, therefore, be that augmented endometrial angiogenesis during the menstrual cycle leads to an increased vessel density in the decidua and thus negatively influences the capacity of EVTs to affect non-arterial vessels. Compromised venous and lymphatic invasion by EVTs might therefore be the consequence of altered endometrial vascularization.

In summary, we demonstrate that EVTs target and invade decidual veins and additionally show for the first time trophoblast-mediated invasion of lymphatic vessels. Strikingly, we were able to detect cells with EVT-specific gene expression pattern in resected lymph nodes of pregnant women diagnosed with cervical cancer. We further demonstrate that RSA is associated with a reduced ratio of EVT-affected veins and lymphatics in relation to the total number of vessels.

Authors' roles

K.W. and J.P. designed, performed and supervised experiments, prepared the figures, analysed and interpreted data and wrote the manuscript. V.K. performed experiments and provided technical assistance. C.F., C.G., G.K., S.P. and S.D. were involved in sample collection, provided patients information necessary for this study and C.G., G.K., S.D. performed primary histopathological assessment of RSA deciduae and resected lymph nodes. M.K. provided intellectual input and critically revised the manuscript.

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Conflict of interest

None declared.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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