Elastin-derived peptides stimulate trophoblast migration and invasion: a positive feedback loop to enhance spiral artery remodelling.

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Elastin-derived peptides stimulate trophoblast migration and invasion: a positive feedback loop to enhance spiral artery remodelling.

**Running title:** Elastin peptides promote trophoblast invasion

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Abstract

Elastin breakdown in the walls of uterine spiral arteries during early pregnancy facilitates their transformation into dilated, high-flow, low-resistance channels. Elastin-derived peptides (EDP) can influence cell migration, invasion and protease activity, and so we hypothesised that EDP released during elastolysis promote EVT invasion and further elastin breakdown. Treatment of the trophoblast cell line SGHPL4 with the elastin-derived matrixine VGVAPG (1µg/ml) significantly increased total elastase activity, promoted migration in a wound healing assay and increased invasion through Matrigel-coated transwells compared to vehicle control (0.1% DMSO) or the scrambled sequence VVGPA. Furthermore, treatment of first trimester placental villous explants with this EDP significantly increased both the area of trophoblast outgrowth and distance of migration away from the villous tips. Primary first trimester cytotrophoblast exposed to VGVAPG (1µg/ml) for 30 minutes showed increased phosphorylation of eNOS and activation of the MAPK pathway, events also associated with tumour cell migration and invasion. These in vitro observations suggest liberation of bioactive EDP during induction of elastolysis in the uterine spiral arteries may orchestrate a positive feedback loop that promotes EVT invasion and further elastin breakdown, contributing to the process of vascular remodelling.

Key Words: Elastase activity / Extravillous Trophoblast / First-trimester / Human placenta / Intracellular signalling
Introduction

Remodelling of the uterine spiral arteries during the first twenty weeks of pregnancy ensures maternal blood is delivered to the placenta at rate of flow that allows it to meet an increasing requirement for nutrients and oxygen, and enables the developing fetus to attain its full growth potential (Burton et al. 2009). Incomplete remodelling leads to poor placental perfusion, a recognised feature of pre-eclampsia (Kadyrov et al. 2003; Pijnenborg et al. 2006), which affects 2-8% of pregnancies and is a major cause of maternal and perinatal morbidity and mortality worldwide (Ghulmiyyah and Sibai 2012). However, the etiology of pre-eclampsia remains poorly understood and delivery of the placenta is currently the only effective treatment. It is therefore important to understand the regulation of spiral artery remodelling and identify possible therapeutic targets.

In normal pregnancy, the process of uterine spiral artery remodelling involves extravillous trophoblasts (EVT), which detach from anchoring placental villi and invade the decidua and myometrium of the uterine wall (interstitial invasion) or migrate through the uterine spiral arteries as far as the inner third of the myometrium (endovascular invasion). The interaction of EVT with the spiral arteries results in specific changes to vessel wall structure (Kaufmann et al. 2003), with temporary loss of vascular endothelium and disappearance of underlying vascular smooth muscle cells (VSMC) (Ashton et al. 2005; Smith et al. 2009); VSMC are replaced by EVT embedded in an amorphous fibrinoid matrix (Pijnenborg et al. 1983). The molecular triggers of remodelling are not known, but specific "trophoblast-independent" and "trophoblast-dependent" stages can be identified (Smith et al. 2009).

Catabolism and reorganisation of the vessel extracellular matrix (ECM) is also fundamental for successful spiral artery transformation. ECM components found in the arterial wall...
include collagen, elastin, glycoproteins (e.g. laminin), and proteoglycans. During the
remodelling process, the internal elastic lamina and musculo-elastic media of the spiral
arteries are degraded, a prerequisite for the formation of dilated, high-flow, low-resistance
channels that lack vasomotor control (Khong et al. 2003; Harris and Aplin 2007). As
incomplete arterial remodelling observed in pre-eclampsia is characterised by the persistence
of narrow bore arteries retaining elastic, muscular walls, particularly within the myometrium
(Khong et al. 1986), we hypothesized that degradation of elastin represents a rate limiting
step during vascular transformation. There is evidence to suggest that uterine natural killer
cells (uNK) and macrophages, which mediate trophoblast-independent remodelling events
prior to the arrival of EVT (Robson et al. 2012), produce enzymes capable of elastolysis
(Craven et al. 1998; Smith et al. 2009). However, uNK cells are uncommon in the
myometrium (Pijnenborg 2002), and elastin breakdown is positively associated with the
presence of endovascular EVT (Pijnenborg et al. 1999), rather than macrophages (Reister et
al. 1999). First-trimester EVT synthesize and secrete the elastolytic proteases matrix
metalloproteinase-2 (MMP-2), MMP-7, MMP-9, MMP-12, cathepsin B, and cathepsin L
(Laszlo et al. 1990; Staun-Ram et al. 2004), and can engulf elastin fibres in vitro (Harris et al.
2010). It is also likely that VSMC-derived elastases contribute to the remodelling process, as
cultured vascular VSMC display robust elastase activity (Harris et al. 2010). Thus we
propose that EVT, VSMC, uNK cells and macrophages cooperatively effect elastolysis in
remodelling spiral arteries, and that EVT- and VSMC are more influential in the myometrial
vessel segments where immune cells are sparse. Understanding the mechanisms underlying
EVT invasion and elastolysis within the myometrial spiral arteries will be helpful to our
understanding of the pathogenesis of pre-eclampsia.
Elastin contains hydrophobic domains rich in nonpolar amino acids such as glycine (G), valine (V), proline (P), and alanine (A), which occur in repeats of three to six peptides such as GGVP, GVGVP, or VGVAPG. As previously observed in other remodelling tissues including the skin, lung and atherosclerotic arteries (Houghton et al. 2006; Almine et al. 2012; Maurice et al. 2013), we hypothesize that elastolysis in the spiral arteries by EVT, VSMC, and uNK cell elastases will release these elastin-derived peptides (EDP). Short bioactive peptides liberated by partial proteolysis of ECM are termed “matrikines” (Maquart et al. 2004), and the hexapeptide VGVAPG is well known for its chemotactic activity towards monocytes, fibroblasts and tumour cells, and its ability to upregulate MMP expression and activity (Duca et al. 2004). MMP-9 and MMP-12-mediated cleavage of elastin can generate VGVAPG (Taddese et al. 2009; Heinz et al. 2010), and EVT, VSMC, uNK cells and macrophages are sources of these enzymes (Naruse et al. 2009; Smith et al. 2009; Harris et al. 2010; Hazan et al. 2010; Anacker et al. 2011). We propose that initiation of ECM catabolism during spiral artery remodelling may orchestrate a positive feedback loop through the generation of EDP to promote invasion and further elastin breakdown within the myometrial vessel segments. Here we test the hypothesis that the matrikine VGVAPG enhances VSMC and EVT elastase activity and migration, and promotes EVT invasion.
Materials and Methods

Ethical Approval: First-trimester placenta (6–10 weeks gestation) was obtained after surgical or medical termination of pregnancy for psychosocial reasons. Tissue was obtained with written informed consent, as approved by the North West Research Ethics Committee (REC Ref: 08/H1010/28). Human placental tissue was used in accordance with the principles set out in the Declaration of Helsinki.

Materials: Unless stated otherwise, all other materials used were obtained from Sigma-Aldrich (Poole, UK).

Methods:

Preparation of elastin-derived peptide

Peptide sequences VGVAPG (representing bioactive EDP) and VVGPGA (a scrambled sequence included as a negative control) were synthesised by Insight Biotechnology, UK. Peptides were reconstituted to a concentration of 1mg/ml in DMSO and then diluted in culture medium. EDP occur in human blood in a wide range of concentrations, from $10^{-6}$ to $10^{-2}$mg/ml (Fulop et al. 1990). Here we examined the effects of 0.1-10µg/ml EDP assuming higher concentrations within this physiological range are present locally in vivo at the site of active spiral artery remodelling. A vehicle control (0.1% DMSO) was included in all experiments.

Cell Culture

SGHPL-4 cells (derived from primary human first trimester extravillous trophoblasts transfected with the early region of SV40, previously known as MC418) were cultured in Ham’s F10 medium. Human aortic SMCs (HASMCs) were cultured in Kaighn’s modification
of Ham’s F12 medium. Medium was supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 µg/ml). First trimester primary CTBs were cultured in 1:1 Dulbecco’s modified Eagle’s medium/Ham’s F12 supplemented with 10% FBS, L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 µg/ml).

Isolation of primary CTBs was performed as previously described (Harris et al. 2006). CTB isolates were plated onto Matrigel-coated plastic (BD Biosciences, San Jose, CA, USA; diluted to 1µg/ml in serum-free medium) to promote a more advanced extravillous phenotype. All cells were incubated with 95% air and 5% carbon dioxide at 37°C in a humidified incubator.

Total Elastase Activity Assay

Following culture of SGHPL-4 cells and HASMCs in the presence or absence of peptides, cell lysates were generated by treatment with 0.1% (vol/vol) Triton X100 in PBS for 30 min. This process induced cell lysis, release of intracellular elastases, and dissociation of membrane-associated elastases. Extracts were centrifuged to remove any cellular debris, and the resulting supernatant, which contained both intracellular and membrane-associated elastases, was retained for analysis. No elastase activity was detected in the pellets. Supernatants (50 µl) were incubated with 150 µl of N-succinyl-(L-alanine)3-p-nitroanilide (1 mmol/L) dissolved in Tris-HCl (200 mmol/L; pH 8.0) for 2 hours at 37°C in a 96-well plate. On cleavage by elastase this substrate absorbs at 405 nm. After addition of 5 µl of glacial acetic acid, the A405 of each sample was determined and compared to a calibration curve prepared using porcine pancreatic elastase. The protein concentration of each sample was measured using a BioRad protein assay with a standard curve prepared using bovine serum albumin. Elastase activity data were expressed as activity per mg of protein.
Migration assay

SGHPL-4 cells were seeded into 6-well plates (2.0-2.5 x 10^5 cells/well). After 24 hours when the cells had reached confluence, a wound area was carefully created by scraping a cross in the cell monolayer with a sterile 200µl pipette tip. The wounded cell monolayer was then washed twice with medium to remove any floating cells or cell debris and photographed using an inverted microscope. Four fields of view were captured per treatment; north, south, east and west of the cross point. The cells were cultured for a further 24 h in the presence of absence of peptide, before being photographed again. Cell migration was assessed by counting the number of cells that had moved into the wound area.

Matrigel invasion assay

Cell invasion was determined by the ability of cells to cross Matrigel-coated polycarbonate membrane Transwell inserts (8 and 12 µm pore size for SGHPL-4 and primary CTBs respectively; Costar, Costar, Cambridge, MA, USA). Briefly, the inserts were coated with a thin layer of Matrigel (1 mg/ml, BD Biosciences, San Jose, CA, USA), achieved by dispensing 30 µl onto the membrane and leaving briefly before removing any excess. Matrigel was allowed to gel at 37 °C for at least 30 min. Cells were placed in the upper chamber (5 x 10^4 and 6.5-7.5 x 10^4 cells per insert for SGHPL-4 and primary CTBs respectively, in 500µl medium), while the lower chamber was loaded with 500 µl culture medium. After overnight culture at 37 °C to allow cells to adhere, the medium was replaced and cells were cultured in the presence or absence of peptide.

Immunocytochemistry of invading cells

After 24 hours (SGHPL-4) or 48 hours (primary CTBs) of culture in Transwell inserts, non-invasive cells on the upper surface of the filter were gently wiped away with a cotton-tipped
swab, and cells on the lower surface were fixed in 4% (vol/vol) paraformaldehyde in PBS.

SGHPL-4 cells were stained with hematoxylin for 10 min at room temperature whereas primary CTBs were immunostained for cytokeratin 7 to confirm their identity as follows: cells were washed with PBS then incubated for 10 min with 3% (v/v) H$_2$O$_2$ in distilled water to quench endogenous peroxidase. Following a second wash step, cells were incubated at room temperature for 30 min with blocking solution (5% BSA in PBS). Primary antibody (monoclonal mouse anti-human cytokeratin 7 Clone OV-TL 12/30; Dako; 1:500 dilution in PBS) was then applied overnight at 4°C. Unbound primary antibody was removed by washing with PBS. Cells were incubated for 30 min at room temperature with biotinylated goat anti-mouse secondary antibody (Dako, diluted 1:200 in PBS), washed again with PBS, and then incubated for a further 30 min at room temperature with avidin-peroxidase. Following another wash with PBS, cytokeratin 7 was detected by colour development with diaminobenzidine-hydrogen peroxide (DAB) and the cells were counterstained with Harris’ haematoxylin. Membranes were mounted onto glass slides using CC/Mount aqueous mounting medium. The number of invading cells was quantified by counting four random fields.

**TUNEL**

SGHPL-4 cells were seeded onto glass coverslips in 12-well plates (5 x 10$^4$ cells/well), allowed to adhere and cultured in the presence or absence of peptides for 24h. Cells were fixed using 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, washed in PBS (2 X 5 min) and labelled with an *In Situ* Cell Death Detection kit (TUNEL; Roche): cells were treated with permeabilisation solution (0.1% (v/v) Triton in 0.1% (w/v) sodium citrate in H$_2$O; 8 min), washed in PBS (3 X 5 min), and covered with 50 µl of TUNEL reagent, which was prepared according to the manufacturer’s instructions, although the TUNEL
enzyme provided was diluted 1:5 with PBS to reduce background fluorescence. Coverslips were incubated in a humidified chamber at 37°C for 1 h in the dark, then were washed in PBS (3 X 5 min). Coverslips were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories) and were stored at 4°C in the dark. Five randomly selected fields of view were captured using a Zeiss AxioVision fluorescence microscope. Quantification of the number of TUNEL-positive cells was performed blind; the number of positive/negative cells in each image were counted and used to determine a mean percent of TUNEL positive cells per coverslip.

**EDP-mediated intracellular signalling profiling in primary first-trimester trophoblast**

Phosphorylation of intracellular proteins following EDP treatment was determined using a Human Phospho-Kinase Antibody Array Kit (R&D Systems) following manufacturer’s instructions. Freshly isolated primary CTBs were plated onto Matrigel-coated plastic to promote an extravillous phenotype and cultured overnight at 37 °C. Medium was replaced and cells were exposed to either 1µg/ml EDP or 0.1% DMSO (vehicle control) for 30 min before lysing in buffer supplied with the kit. The protein concentration of each sample was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). Lysates from 3 separate cell preparations were pooled for analysis (70µg protein from each) and incubated overnight with the membranes provided. The Array kit utilises a streptavidin-HRP and chemiluminescent detection system. Image J software was used to quantify signal intensity.

**Extravillous trophoblast outgrowths**

First-trimester placental tissue (6–10 weeks gestation) was washed in warmed 1:1 mix of DMEM and Ham's F12 containing L-glutamine (2 mmol/L), penicillin (100 IU/ml), and streptomycin (100 µg/ml). Terminal portions of villi with trophoblast cell columns were selected and separated under a dissecting microscope as previously described (Aplin et al.)
Villous tips were seeded onto collagen gels prepared in advance using 1ml of rat tail collagen (4mg/ml; BD Biosciences, Oxford, UK) mixed with 50µl of serum-free culture medium, followed by 100µl of 7.5% NaHCO₃. As the collagen polymerised, 100µl was aliquoted into each well of a 24-well culture plate and left to set at 37°C for 30min. One villous tip was transferred to the gel surface using fine forceps, surrounded by culture medium, and incubated overnight with 95% air and 5% carbon dioxide at 37°C in a humidified incubator. Attachment was confirmed at ~16 h by the appearance of radial stress lines in the collagen gel, at which point 1ml of medium (+/- peptide) was added to cover each villous tip before photographing using a Nikon Coolpix 990 digital camera and Nikon Eclipse TE 200 inverted microscope (Nikon, Tokyo, Japan). Villi were photographed again at 24 and 48 h. Each villous explant, in the process of being assessed and photographed, was exposed to ambient air outside the incubator for up to 30 min. Overlapping images from each time point were aligned using Microsoft Powerpoint, and trophoblast outgrowth area and distance migrated (mean of 6 trajectories per explant) were measured using ImagePro Plus software. The same explant was assessed serially throughout the culture period allowing changes in outgrowth and migration to be recorded.

**Placental explant culture**

First trimester mesenchymal villous placental fragments (2-3 mm³) were dissected under sterile conditions in serum-free DMEM/F12 and transferred to 24-well tissue culture plates pre-coated with 1% (w/v) agarose. Explants were maintained in serum-free DMEM/F12 in the presence or absence of peptide for 24 h in 20% O₂ at 37°C.

**Immunohistochemistry of placental explants**
First trimester placental villous explants were fixed in 10% neutral buffered formalin overnight at 4°C before thorough washing in Tris buffered saline (TBS) and paraffin embedding. Tissue sections (5µm) were dewaxed and rehydrated before antigen retrieval was performed by microwaving the slides for 2 x 5 min at 800W in 0.01M citrate buffer (pH6.0). Non-specific staining was prevented by quenching endogenous peroxidase activity with 3% aqueous H$_2$O$_2$, and was followed by a 30 min blocking step with 5% (wt/vol) BSA in TBS. Primary antibodies, diluted to working concentration with 0.05 mol/L TBS (phosphorylated eNOS (phospho- S1177), 1:200, Abcam, UK; phosphorylated p38 MAPK (phospho-Thr180/Tyr182), 1:100, Cell Signalling, UK; phosphorylated ERK (phospho-Thr202/Tyr204), 1:200, Cell Signaling, UK; control IgG, 1:1000, Sigma Aldrich, UK) were applied to the tissue sections, which were incubated overnight at 4°C in a humidity chamber. Following repeated washes with TBS, antibody binding was detected by sequential application of biotinylated swine anti-rabbit IgG (Dako: Ely, UK. 1:200 dilution) and avidin-peroxidase (20ng/ml), with the chromogen diaminobenzidine, to produce a brown precipitate. Sections were counterstained with Harris’ haematoxylin, dehydrated and mounted with DPX. Images of EVT columns were captured using a Leitz 22 microscope in conjunction with a QI Cam Fast 1394 camera and Image ProPlus software. Staining intensity was assessed by two independent blinded observers using a scale of 0-4.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism software version 5 (GraphPad Software, San Diego, CA). Nonparametric data are represented by median and range and was analyzed using a Kruskal–Wallis test with appropriate post hoc analysis. **Values in the Results text refer to the median.** Data represent at least three independent experiments. Significance was taken as $P \leq 0.05$. 

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Results

Differential response of trophoblast and HASMCs to VGVAPG

To investigate whether biologically active EDP promote events involved in the process of spiral artery remodelling, we analysed trophoblast and HASMC elastase activity and migration following incubation with VGVAPG (0.1-10µg/ml), as compared to vehicle control (0.1% v/v DMSO). Elastase activity in SGHPL-4 was increased following incubation for 48h with 1µg/ml VGVAPG (0.056µg/mg vs 0.007µg/mg in vehicle controls, p<0.05; Figure 1a). Incubation of SGHPL-4 cells with 1µg/ml VGVAPG for 24h increased repopulation of the denuded area of a wounded cell monolayer (38 migrated cells vs 15 migrated cells in vehicle controls, p<0.05; Figure 1b). Transwell invasion of SGHPL-4 was also significantly increased following 24h incubation with 0.5µg/ml and 1µg/ml VGVAPG (12 and 17 invasive cells respectively vs 4 invasive cells in vehicle controls, p<0.05 and p<0.01 respectively; Figure 1c); the scrambled peptide VVGPGA had no effect. The bell-shaped curves obtained in these assays indicate that effects diminish at higher VGVAPG concentration. A 3 fold increase in the number of TUNEL positive cells was observed following incubation of SGHPL-4 cells with 10µg/ml VGVAPG compared to all other treatments (Figure 1d). This observation indicates the decreased effects of VGVAPG at higher concentrations on active cell processes may be due to onset of apoptosis.

VGVAPG did not enhance elastase activity or migration of HASMC at any of the concentrations tested (Figure 2). The scrambled peptide VVGPGA (1µg/ml), which was included as a negative control, did not significantly alter migration or elastase activity.

These experiments established that the concentration of VGVAPG that induced a maximal biological response was 1µg/ml. Therefore, this concentration was used for subsequent experiments using first trimester primary CTBs and placental villous explants.
VGVAPG enhance invasion and migration of primary first-trimester EVT

Invasion assays across Matrigel-coated Transwell inserts were carried out using first trimester CTB exposed to VGVAPG for 48h (Figure 3a). Cytokeratin 7 immunostaining of cells that had crossed the insert membrane confirmed their identity as trophoblasts. Invasion increased following VGVAPG treatment in all 5 experiments performed (47 invasive cells vs 30 invasive cells in vehicle treated controls) but the data did not reach statistical significance. However, in a more physiologically relevant model, EVT migration from trophoblast cell columns of placental villous explants (Figure 3b,c) was significantly increased following 24h incubation with VGVAPG (1µg/ml). There was a significantly greater increase in both EVT outgrowth area (61µm² vs 11 µm²) and distance migrated from the villous tips (5.2µm vs 1.4µm) compared to explants cultured with vehicle control (0.1% v/v DMSO; Figure 3b: p<0.01, and Figure 3c: p<0.05 respectively).

Identification of VGVAPG-mediated intracellular signalling events in primary first-trimester EVT

To detect intracellular signalling pathways initiated by VGVAPG, site-specific phosphorylation of 43 kinases and 2 related total proteins was compared by antibody array following acute exposure (30 minutes) of primary first-trimester EVTs to VGVAPG (1µg/ml) or vehicle control (0.1% v/v DMSO). As shown in Table 1, phosphorylation of eNOS was increased just over 3-fold. In agreement with this observation, immunohistochemical analysis of first trimester explants treated for 24 hours with VGVAPG (1µg/ml) demonstrated an increase of phosphorylated eNOS compared to vehicle control (Figure 4). Phosphorylation of Akt, a molecule upstream of eNOS, was unaffected after 30 minutes of exposure to VGVAPG (Table 1). Examining earlier time points following EDP treatment is likely to reveal increased phosphorylation of Akt. VGVAPG also increased phosphorylation in the
mitogen activated protein kinase (MAPK) pathway of targets that included p38α, MEK 1/2, and MSK 1/2 (Table 1 and Figure 4). Phosphorylation of effectors further down the pathway, such as ERK 1/2 and p53, was unaltered after 30 minutes of exposure to VGVAPG; immunohistochemical analysis of first trimester explants confirmed that VGVAPG treatment for 24 hours did not increase ERK phosphorylation (Figure 4). β-catenin, a signal transducer in the Wnt pathway, was also increased by VGVAPG; upstream of β-catenin however there was decreased phosphorylation of GSK3α/β. Other signalling proteins with decreased phosphorylation were TOR and Src (Table 1). Phosphorylation of JAK/STAT was unaffected by VGVAPG, suggesting this pathway is not involved in initiation of signal transduction following exposure of EVT to EDP.
Discussion

Our data demonstrate that the elastin-derived peptide VGVAPG promotes EVT migration and invasion in vitro, and that the biological effects of VGVAPG are mediated via phosphorylation of eNOS and activation of the MAPK pathway. This is the first study to demonstrate a role for a matrikine in the regulation of EVT behaviour in human pregnancy and our findings suggest that induction of ECM elastolysis during spiral artery remodelling could establish a positive feedback loop that promotes deeper EVT invasion of the myometrium and further elastin breakdown.

Promotion of EVT invasion, migration and elastase activity by EDP has previously been reported in other cell types, including fibroblasts, macrophages and tumour cell lines (Senior et al. 1982; Maeda et al. 2007; Pocza et al. 2008; Coquerel et al. 2009). The bell-shaped response to VGVAPG seen in SGHPL-4 cells has also been observed in other cell types (Senior et al. 1982; Shiratsuchi et al. 2010), and may be explained by our finding that higher concentrations of VGVAPG induce apoptosis. Indeed, it has been previously shown that concentrations of EDP above 1µg/ml can induce cell death in lymphocytes (Peterszegi et al. 1999). If these observations hold true in vivo, they suggest that high local EDP concentrations could halt EVT invasion and act as an efficient regulatory system to limit further ECM breakdown and maintain blood vessel integrity during the remodelling process. EDP have also been shown to stimulate proliferation of human skin fibroblasts (Shiratsuchi et al. 2010); however, in preliminary studies, proliferation of cytotrophoblast in first trimester placental explants was unaffected by VGVAPG treatment (data not shown).

VGVAPG did not influence HASMC elastase activity and migration in vitro. We have previously shown elastase activity in cultured HASMC (Harris et al. 2010); however, MMP-
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12 expression in human spiral artery segments was upregulated by EVT-conditioned medium (Harris et al. 2010), suggesting that soluble factors released by EVT, rather than EDP, upregulate VSMC elastase activity. Thus, resident VSMC may initiate elastin breakdown in distal myometrial spiral arteries prior to EVT colonisation, or alternatively, may cooperate with EVT to achieve efficient elastin catabolism. Our data also suggest that migration of VSMC into the decidual stroma during the remodelling process is not regulated by EDP; as elastin is virtually absent from decidual arterioles (unpublished observations), the concentration of EDP within the decidua is likely to be minimal.

Studies in a wide range of cell types, not including the placenta, have shown that the effects of EDP are mediated by three cell surface receptors: integrin αvβ3, galectin 3 (Gal3), and elastin receptor/elastin binding protein complex (ERC) (Pocza et al. 2008). At present our analysis of receptor function in the response of trophoblast to VGVAPG is incomplete because analytical tools are still in development, but we discuss briefly here the candidate pathways. As integrin αvβ3 is expressed on trophoblasts within cell columns, and on the surface of interstitial and endovascular EVT (Zhou et al. 1997) this may act as a receptor for EDP in trophoblast. One study has reported that Gal3 is also highly expressed in EVT cell columns of anchoring villi but is absent from invasive interstitial EVTs during the first trimester (Vicovac et al. 1998); however, another has demonstrated Gal3 expression in cell columns and in interstitial and endovascular EVT (Maquoi et al. 1997). Despite this controversy, Gal3 protein expression in primary cytotrophoblast isolated from first trimester placenta was predominantly intracellular (Kolundzic et al. 2011). Therefore it is unlikely that EDP mediates a biological response via Gal3 in EVT. Functional studies in the JEG3 choriocarcinoma cell line show ERC knockdown significantly inhibits basal invasion and
migration (Wang et al. 2013), supporting a role for the ERC as a mediator of EDP-stimulated
trophoblast invasion.

EDP signalling can occur via a number of pathways depending on cell type and the receptor
involved. In the current study, eNOS was the most highly phosphorylated protein following
acute VGVAPG treatment of EVT, along with components of the MAPK pathway including
p38α, MEK 1/2, and MSK 1/2. Phosphorylation downstream of MAPK, in ERK 1/2 and p53,
were unaffected after 30 minutes’ exposure to VGVAPG. Increased phosphorylation of
eNOS and p38 MAPK persisted after 24 hours’ treatment with VGVAPG, suggesting these
signalling events are relevant to the functional assays performed at the same time point.

eNOS and MAPK signalling have been shown to mediate EDP effects in other non-placental
cell types (Robinet et al. 2007; Garczorz et al. 2011) and are also regulators of tumour cell
migration (Jadeski et al. 2003).

eNOS is expressed in EVT (Martin and Conrad 2000) and previous studies have shown that
nitric oxide (NO) production can influence MMP activity (Brown et al. 2004; McCarthy et al.
2008) and regulate trophoblast function (Harris et al. 2008). EDP-mediated MMP production
occurs via ERK1/2 in skin fibroblasts (Duca et al. 2007) and activation of MMP in other cell
types is also regulated by MAPK (Dodd et al. 2011). In a similar timeframe to our
observations, EDP have been shown to trigger NO production in human endothelial cells via
the PI3K/Akt/eNOS pathway after a 20 minute exposure, leading to an increase in MMP
expression via MEK 1/2 (Fahem et al. 2008; Garczorz et al. 2011). Thus the observed
increases in EVT migration and invasion observed in our study are likely to correlate, at least
in part, to the observed increases in elastase activity.
Akt-dependent phosphorylation of mTOR is thought to play a critical role in trophoblast cell migration (Busch et al. 2009). However, we found no evidence for activation of this pathway by VGVAPG in EVT. VGVAPG treatment of EVT led to a loss of GSK3 phosphorylation on serine residues -9 in GSK3β and -21 in GSK3α. Phosphorylation of these sites inhibits the activity of GSK3 (Jope et al. 2007) suggesting VGVAPG increases GSK3 activity in EVT. Both GSK3α and GSK3β are in an activated state during the most active phase of epithelial cell migration (Farooqui et al. 2006). However, VGVAPG also increased β-catenin, a protein which is degraded through the action of GSK3. Degradation of β-catenin requires pre-phosphorylation by a priming kinase and association of scaffolding proteins before GSK3 can act. It is therefore possible that this β-catenin destruction complex was not adequately assembled and this would explain our conflicting observations. Squamous cell carcinoma overexpressing β-catenin exhibit significantly higher invasion/migration capacity (Iwai et al. 2010).

Our previous work has implicated MMP-12 as the major elastase utilised by EVT (Harris et al. 2010). As MMP-12-mediated cleavage of elastin generates EDP containing XGXXPG motifs (Taddese et al. 2009), trophoblasts are equipped to produce these bioactive peptides as well as respond to them. Therefore, we believe that in healthy pregnancy, products of elastin catabolism further enhance EVT invasion and elastolysis in the spiral arteries. As elastin catabolism is impaired in the myometrial spiral arteries in pregnancies complicated by preeclampsia (Khong et al. 1986), we predict that elastase activity and EDP release is also impaired. Interestingly, first trimester chorionic villous biopsies from women who go on to develop pre-eclampsia showed a dramatic reduction in MMP-12 mRNA expression (Founds et al. 2009). Furthermore, ERC expression (Kurdoglu et al. 2011) and eNOS activity is reduced in placentas from pre-eclampsia compared to healthy controls (Morris et al. 1995;
Xiang et al. 2005). As EDP have been shown to be protective against ischemia/reperfusion injury in an ex vivo heart model (Robinet et al. 2007), reduced EDP formation in pre-eclampsia could potentiate the damaging effects of ischemia at the maternofetal interface.

Our data support a model whereby breakdown of elastin within myometrial spiral arteries liberates EDP that promote elastase activity, migration, and invasion of EVT. This feedback loop could have an important role in the successful completion of spiral artery remodelling during early pregnancy. Other matrikines could have similar roles; the laminin sequence LGTIPG as well as peptides from type IV collagen both enhance cell migration (Pasco et al. 2004). There is evidence to suggest generation of collagen XVIII fragments by trophoblast-derived proteases could play a role in the regulation of trophoblast invasiveness (Pollheimer et al. 2005). Due to their bioactivity toward many different cell types, matrikines have been identified as playing a major role in regulating both physiological and pathological processes. Further research to establish the importance of these peptides in the process of uterine spiral artery remodelling and the implications for development of pre-eclampsia could contribute to our understanding of disease development.
Authors Declaration

M.D., L.K.H., and J.D.A. conceived all of the experiments. M.D. and L.K.H. carried out all of the experiments. The manuscript was prepared by M.D and all authors critically revised the manuscript and approved the final version.

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Funding

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Conflict of Interest

No conflicts of interest are declared.
**Figure Legends**

**Figure 1.** EDP treatment of SGHPL-4 cells increases **a)** elastase activity, **b)** migration, and **c)** invasion. **a)** Elastase activity in cell supernatants was assessed using the substrate N-succinyl-(L-alanine)₃-p-nitroanilide, which absorbs strongly at 405nm upon cleavage. **b)** Number of migrating cells into the denuded area of a wound healing assay. **c)** Number of invading cells assessed using a Transwell assay. Line represents median, n=5: * p<0.05, ** p<0.01 vs 0.1% DMSO vehicle control; # p<0.05 ## p<0.01 vs 10µg/ml EDP VGVAPG; + p<0.05 vs 1µg/ml scrambled sequence VVGPGA. Kruskal-Wallis with Dunn’s post hoc test. **d)** Percentage of SGHPL-4 cells that are TUNEL-positive. Includes DMSO vehicle controls: 0.1% (V-low) and 1% (V-high). Line represents median, n=3.

**Figure 2.** EDP does not affect HASMC **a)** elastase activity and **b)** migration. **a)** Elastase activity in cell supernatants was assessed using the substrate N-succinyl-(L-alanine)₃-p-nitroanilide, which absorbs strongly at 405nm upon cleavage. **b)** Number of migrating cells into the denuded area of a wound healing assay. Vehicle: 0.1% DMSO, VVGPGA: scrambled sequence, VGVAPG: EDP. Line represents median, n=4.

**Figure 3.** EDP enhances invasion and migration of first trimester extravillous trophoblast. **a)** Transwell invasion of cytokeratin 7 positive cells (brown) and number of invading cells following 48hr treatment with 1µg/ml EDP VGVAPG or vehicle (0.1% DMSO). Line represents median, n=5. **b&c)** Extravillous trophoblast outgrowths from first-trimester villous explants following 24hr treatment with 1µg/ml EDP VGVAPG or vehicle (0.1% DMSO). **b)** Outgrowth area (outlined in red), n=5, and **c)** distance migrated (red arrow lines), n=4, were calculated using Image ProPlus software. * p<0.05, ** p<0.01 vs vehicle. Line represents median, Mann Whitney U test.

**Figure 4.** Immunohistochemical analysis of EDP-mediated intracellular signalling in EVT. **a)** Expression of phosphorylated eNOS, phosphorylated p38 MAPK, and phosphorylated ERK was examined using the streptavidin-biotin-peroxidase complex technique in formalin-fixed, paraffin-
embedded sections from first trimester villous explants cultured for 24h with 1µg/ml EDP VGVAPG (right hand column) or vehicle control (0.1% DMSO; left hand column). Inset: Rabbit IgG negative control. b) The intensity of staining in EVT cell columns was scored between 0 and 4, using a semiquantitative scale (n=3).

**Table 1.** EDP-mediated intracellular signalling in primary first-trimester EVT.

Fold change in the relative phosphorylation of selected signalling molecules following 30 minutes exposure to 1µg/ml EDP VGVAPG compared to vehicle controls. Pooled samples, n=3.
References


Dodd T, Jadhav R, Wiggins L, Stewart J, Smith E, Russell JC and Rocic P. MMPs 2 and 9 are essential for coronary collateral growth and are prominently regulated by p38 MAPK. J Mol Cell Cardiol 2011; 51(6): 1015-25.


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Figure 1

394x868mm (300 x 300 DPI)
Figure 2

(a) Elastase activity (µg/mg protein) vs. VGVAPG (µg/ml)

(b) Number of cells migrating into wound after 24h vs. VGVAPG (µg/ml)
Figure 3

a) Vehicle

VGVAPG

Number of invading cells

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

Vehicle
VGVAPG µg/ml

b) Increase in outgrowth area (µm²)

90
80
70
60
50
40
30
20
10
0

Vehicle
VGVAPG µg/ml

+ 24hrs

Increase in outgrowth distance (µm)

6
5
4
3
2
1

Vehicle
VGVAPG µg/ml

+ 24hrs

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Figure 4

a) Vehicle  VGVAPG 1μg/ml

- Phospho eNOS
- Phospho 388
- Phospho ERK

b) Graphs showing staining intensity scores for phospho-eNOS and phospho-ERK under vehicle and VGVAPG 1μg/ml conditions.

192x178mm (300 x 300 DPI)
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<th>Target protein</th>
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<td>eNOS</td>
<td>S1177</td>
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