Proprotein convertase 5/6 cleaves platelet-derived growth factor A in the human endometrium in preparation for embryo implantation

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**ABSTRACT:** Establishment of endometrial receptivity is vital for successful embryo implantation. Proprotein convertase 5/6 (referred to as PC6) is up-regulated in the human endometrium specifically at the time of epithelial receptivity. PC6, a serine protease of the proprotein convertase family, plays an important role in converting precursor proteins into their active forms through specific proteolysis. The proform of platelet-derived growth factor A (pro-PDGF-A) requires PC cleavage to convert to the active-PDGF-A. We investigated the PC6-mediated activation of PDGF-A in the human endometrium during the establishment of receptivity. Proteomic analysis identified that the pro-PDGF-A was increased in the conditioned medium of HEC1A cells in which PC6 was stably knocked down by small interfering RNA (PC6-siRNA). Western blot analysis demonstrated an accumulation of the pro-PDGF-A but a reduction in the active-PDGF-A in PC6-siRNA cell lysates and medium compared with control. PC6 cleavage of pro-PDGF-A was further confirmed in vitro by incubation of recombinant pro-PDGF-A with PC6. Immunohistochemistry revealed cycle-stage-specific localization of the active-PDGF-A in the human endometrium. During the non-receptive phase, the active-PDGF-A was barely detectable. In contrast, it was localized specifically to the apical surface of the luminal and glandular epithelium in the receptive phase. Furthermore, the active-PDGF-A was detected in uterine lavage with levels being significantly higher in the receptive than the non-receptive phase. We thus identified that the secreted PDGF-A may serve as a biomarker for endometrial receptivity. This is also the first study demonstrating that the active-PDGF-A localizes to the apical surface of the endometrium during receptivity.

**Key words:** endometrial receptivity / implantation / platelet-derived growth factor A / protein convertase 5/6 / proteomics

**Introduction**

Embryo implantation involves stable attachment of a blastocyst to the uterus. The endometrial surface is the first point of contact between the blastocyst and the uterus. In women, the endometrium is receptive to blastocyst attachment only during Days 20–24 of the menstrual cycle; at other times the endometrium is non-receptive (Salamonsen et al., 2009). To change from a non-receptive to a receptive state, the endometrium undergoes substantial structural and functional changes, involving alterations in growth factors, cell surface architecture, adhesion molecules, as well as cell polarity and cytoskeleton-membrane interactions (Denker, 1993; Carson et al., 2000).

Proprotein convertases (PCs) are a family of Ca²⁺-dependent serine endoproteases that post-translationally activate a large number of proproteins (Seidah and Chretien, 1997; 1999; Seidah et al., 2008). In mammals, there are seven basic-amino acid specific PC members (furin, PC1/PC3, PC2, PC4, PC5/6, PACE4 and PC7/PC8) (Seidah et al., 2008). Known targets of PCs include pro-growth factors, peptide hormones, neuropeptides, extracellular matrix proteins, adhesion molecules, proteolytic enzymes and integral membrane proteins (Seidah and Chretien, 1999). PCs are thus regarded as critical ‘master switch’ molecules (Seidah and Chretien, 1999; Rockwell and Thorner, 2004; Scamuffa et al., 2008). In the endometrium, PCs/6 (referred to as PC6) is the only PC member...
that is highly regulated for embryo implantation (Nie et al., 2003, 2005; Tang et al., 2005; Freyer et al., 2007). In mice, PC6 is expressed only in decidual cells and PC6 knockdown causes decidualization inhibition and implantation failure (Nie et al., 2005). Our recent studies have demonstrated that PC6 is critical for endometrial epithelial receptivity in women (Heng et al., 2011a, b). PC6 cleaves crucial scaffolding protein EBPs (Ezrin-binding protein 50), thereby profoundly regulating membrane-cytoskeletal reorganization (Heng et al., 2011a). PC6 also post-translationally activates α-integrins (Paule et al., 2012) and bone morphogenetic protein 2 (Heng et al., 2010) in the endometrium. When PC6 was specifically knocked down by small interfering RNA (PC6-siRNA) in the human endometrial epithelial cell line HEC1A by stable transfection with PC6 siRNA, the cells greatly reduced capacity for blastocyst adhesion (Heng et al., 2011a). Furthermore, PC6 was secreted into the uterine fluid and its concentrations are significantly elevated in the luteal phase in fertile women and markedly reduced in a subgroup of infertile women (Heng et al., 2011a, b).

Platelet-derived growth factor A (PDGFA), a member of the PDGF family, is initially synthesized as an inactive precursor protein (Fredriksson et al., 2004). PDGF is composed of two A (-AA) or two B (-BB) or a combination of A and B (-AB) chains (Ricono et al., 2002; Fredriksson et al., 2004; Seidah et al., 2008). Most epithelial cells express PDGFA and secrete PDGFAA (Heldin et al., 1986; Andrae et al., 2008). The precursor form of PDGFA (pro-PDGFA) is proteolytically cleaved when shuttling through the Golgi apparatus towards the trans-Golgi network and secreted as a dimeric ~30 kDa product (Ostman et al., 1992). The proteolytic processing of pro-PDGFA is essential for the production of the active-PDGFA, which then binds to its receptors (Fredriksson et al., 2004). The active-PDGFA is important in regulating embryonic development, cell proliferation, cell migration, survival and chemotaxis (Beckmann et al., 1988; Clunn et al., 1997; Heldin and Westmark, 1999; Ricono et al., 2002). PDGF and PDGFA have been shown to be important in implantation and placentaion (Persson and Rodriguez-Martinez, 1997; Jaber and Kan, 1998).

Studies in pigs and mice have demonstrated that PDGFA concentrations are high in the uterus at peri-implantation, suggesting a role for PDGFA in the endometrium in preparation for implantation (Persson and Rodriguez-Martinez, 1997; Jaber and Kan, 1998). In the secretory phase, PDGFA mRNA is low in women with endometriosis (Lee et al., 2007). PDGFA is particularly involved in the nutrition and tissue re-organization during placentaion (Persson and Rodriguez-Martinez, 1997; Jaber and Kan, 1998).

Pro-PDGFA contains one strong diabasic PC-cleavage site at amino acid 86 (RRKR86↓). PC member furin was shown to activate PDGFA in the colon carcinoma cell line LoVo (Siegfried et al., 2003). However, the exact PC member that activates pro-PDGFA in the human endometrium is largely unknown. Since PC6 is the only PC up-regulated in the endometrium during the window of receptivity, we hypothesized that PC6 post-translationally cleaves pro-PDGFA and activates this growth factor. In this study, we first compared control and PC6-siRNA HEC1A cell media using proteomics to identify potential substrates of PC6. We then focused on validation of one of the substrates, PDGFA, with a number of approaches to establish the significance of its activation by PC6 in the human endometrium for the establishment of receptivity.

**Materials and Methods**

**Human endometrial tissue collection**

Ethics approval was obtained from the Human Ethics Committee at Monash Medical Centre, Melbourne, Australia. Prior to tissue collection, written informed consent was obtained from all participants. Human endometrial biopsies were obtained from women undergoing curettage for benign conditions. A total of twelve tissues (mid-proliferative phase n = 6, mid-secretory phase n = 6) were fixed in paraffin, embedded in wax and sectioned (5 μm) for immunohistochemistry. For western blot, a total of seven tissues (mid-proliferative phase n = 3, mid-secretory phase n = 4) were used for cell lysis isolation, using a lysis buffer [50 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1% (vol/vol) Triton X-100; 1 mM EGTA; 2 mM EDTA] containing a protease inhibitor cocktail (Pierce, Rochford, IL, USA).

**Uterine lavage collection**

Uterine lavages were obtained as previously described (Heng et al., 2011a, b) from fertile women during proliferative (n = 5) and secretory phase (n = 5) of the menstrual cycle. In brief, in this study 2.2 ml of saline was infused transcervically into the uterine cavity with a fine catheter, the fluid was aspirated, briefly centrifuged (235 g, 5 min), aliquoted and stored at –80°C until use.

**Cell lines**

Human endometrial epithelial HEC1A cells (American Type Cell Culture, Manassas, VA, USA) were stably transfected with a plasmid containing siRNA targeting the human PC6 (hereafter referred to as PC6-siRNA) or a scrambled siRNA counterpart (control), and these stable cell lines were comprehensively characterized previously (Heng et al., 2011a; Paule et al., 2012). The levels of PC6 mRNA (by real-time RT–PCR), protein (by western blot analysis) and activity (in the media by activity assay) were confirmed to be knocked down by ~50% in the PC6-siRNA compared with the control cells (Heng et al., 2011a; Paule et al., 2012). The knockdown was highly specific as no change in the levels of any other PC members was detected. These cells were grown in McCoy’s5a medium (GIBCO BRL, Grand Island, NY, USA) containing G418 (500 mg/ml, Sigma) at 37°C in a humidified and 5% CO2 atmosphere.

Cells were seeded in T75 cm² flasks at a density of ~2 × 10⁶ and cultured for 72 h in 10 ml of McCoy’s5a media with supplements (90% confluent). The media was then removed, and the cells were gently washed twice with 10 ml of phosphate-buffered saline (Invitrogen, Grand Island, NY, USA) and cultured for a further 48 h in serum free McCoy’s5a media with supplements. The conditioned medium from three independent cultures was combined, resulting in 300 μg of total protein (assayed with the BCA kit, Thermo Scientific, Rochford, IL, USA), and was subjected to label-free proteomics as described below.

**Proteomic analysis**

Media (n = 3, pooled into one single sample) from control and PC6-siRNA cells were analysed by proteomics. Proteins were solubilized using sodium dodecyl sulphate (SDS) and digested by the filter-aided sample preparation method (Wisniewski et al., 2009). Briefly, the eluate was added to SDS-containing buffer and loaded onto a Vivaspin 500 30 K device (Sartorius Stedman). SDS was removed by urea exchange followed by dialysis with 50 mM sodium acetamide for 20 min. Urea was then replaced with 20 mM ammonium bicarbonate before the proteins were digested overnight at 37°C with trypsin (Gold, Promega) (1 μg of trypsin per 100 μg of protein). The peptides were collected from the filter by centrifugation followed by two additional elutions with 20 mM ammonium bicarbonate. Peptide eluates were...
then quantified on a Nanodrop 2000 at a wavelength of 280 nm and acidified to 1% formic acid prior to mass spectrometry (MS) analysis.

MS data were acquired on a Q-Exactive™ (QE) hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) fitted with a Nano-ESI source (Proxeon; Thermo Fisher Scientific) coupled to a nanoACQUITY UPLC system (Waters). Approximately 1 μg of total digested peptide was loaded onto a 20 mm long nanoACQUITY pre-column with 180 μm I.D. and 5 μm C18 silica bead (Waters) in 5% buffer B at a flow rate of 10 μl/min (Buffer A—0.1% formic acid, Buffer B—90% ACN, 0.1% formic acid) and then resolved on a 100 mm nanoACQUITY column with 75 μm I.D. and 1.7 μm C18 silica bead (Waters) using a 70 min gradient (5% to 45% buffer B) at a flow rate of 0.4 μl/min. The QE was then run in a data-dependent acquisition mode with the Orbitrap resolution set at 75 000 and the top-ten multiply charged species selected for fragmentation by higher-energy collisional dissociation (single charged species were ignored). The ion threshold was set to 50 000 counts for MS/MS. The CE voltage was set to 27.

Label-free quantification was performed using the MaxQuant software package (Cox and Mann, 2008). High-resolution MS data were searched using a tolerance of 10 ppm for precursor ions and 20 ppm for product ions. Enzyme specificity was tryptic and allowed for up to 2 missed cleavages per peptide. Carbamidomethylation of cysteines was specified as a constant modification with oxidation of methionine and protein N-terminal acetylation set as variable modifications. The MS data were searched against a human protein database (Human_LudwigNR_Q113; 123 036 entries) at a detection limit of 0.85 pg/ml.

**In vitro PC6 cleavage of recombinant full length human PDGFA**

Recombinant full length human PDGFA (80 ng, Abnova, Taipei, Taiwan) was incubated with or without 1 μg recombinant PC6 (PhenoSwitch BioSciences, Inc., Quebec, Canada) in DMEM/F12 at 37°C for 24 h. Samples were subjected to western blot analysis.

**PDGFAA ELISA**

The concentration of PDGFAA in the uterine lavage was determined using Human/Mouse PDGFAA enzyme-linked immunosorbent assay (ELISA) kits (DAA00B, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. This ELISA only recognizes the active, homodimeric form of PDGFA (PDGFAA, communications with R&D systems). Data were analysed using 5 parameter logistic (5-PL) curve-fitting and the minimum detection limit was 0.85 pg/ml.

**Statistical analysis**

Data were expressed as the mean ± SEM. Unpaired Student t-test was used to compare control and PC6-siRNA cells, endometrial cell lysates and uterine lavages, using PRISM version 6 for Windows (GraphPad Software, Inc., San Diego, CA, USA). *P ≤ 0.05 was considered statistically significant and **P ≤ 0.01 highly significant.

**Results**

**Quantitative proteomics identifies significant enrichment of PDGFA in the conditioned medium of PC6-siRNA cells**

Label-free quantitative proteomics allowed us to confidently identify (FDR < 1%) and quantify the relative abundance of 1126 proteins. Based on the Wilcoxon signed-rank test, we identified 16 differently expressed proteins above the threshold (>2-fold change and P-value <0.01) in the conditioned media of PC6-siRNA compared with control cells (Table I). Among these is PDGFA, which was identified to contain a direct PC6 cleavage site (Table I), was previously reported to be activated by another PC family member furin (Siegfried et al., 2003; Haouzi et al., 2011), and was also described as being secreted by the human endometrium (Hannan et al., 2011). We thus focused on the potential role of PC6 in the activation of PDGFA for endometrial receptivity for the remainder of this study. Two unique peptides of PDGFA, TVIYEIPR and LEEHLECACATTSNPDYR, were confidently identified and the corresponding precursor ion intensities found to be consistently higher in each of the three experimental replicates in the conditioned media of PC6-siRNA cells (Fig. 1).

**Western blot analysis validates that pro-PDGFA protein is elevated in the conditioned media of PC6-siRNA cells**

PDGFA can exist as a proprotein or an active growth factor which can be secreted into the conditioned media (Siegfried et al., 2003). To validate...
the proteomic findings, western blot analysis was performed on the conditioned media of control and PC6-siRNA cells with a PDGFA antibody detecting both the pro- and active-PDGFA (Fig. 2). The pro-PDGFA was clearly detected in all cases (Fig. 2A), and the levels were significantly higher in the medium of PC6-siRNA than control cells (Fig. 2C). On the other hand, the active-PDGFA was detected in the control but was below detection in the PC6-siRNA cell medium (Fig. 2A). These data suggest that the processing of PDGFA from its proform to the active form was inhibited in the PC6-siRNA cells.

Processing of pro-PDGFA to its active form is reduced in PC6-siRNA cells lysates

To further confirm the above observation, western blot analysis was performed on the lysates from control and PC6-siRNA cells. While both forms of PDGFA were detected in the lysates using the antibody for the pro- and active-PDGFA (Fig. 3A), the relative amounts of each form were altered in PC6-siRNA compared with control cells (Fig. 3B). On the other hand, the active-PDGFA was detected in the control but was below detection in the PC6-siRNA cell medium (Fig. 2A). These data suggest that the processing of PDGFA from its proform to the active form was inhibited in the PC6-siRNA cells.

PC6 cleaves pro-PDGFA protein in vitro

The likely PC6 cleavage site on the pro-PDGFA protein sequence is RRKR 86↓ (Fig. 4A). Proteolysis at this site would result in the production of the active-PDGFA (monomer, ~16 kDa; dimer ~34 kDa). To confirm that PC6 can cleave human pro-PDGFA, recombinant pro-PDGFA was incubated with recombinant PC6 in vitro, and the products were analysed by western blot with two antibodies, one detecting both the pro- and active-PDGFA (Fig. 4B) and the other detecting only the pro-PDGFA (not shown). The incubation of recombinant PC6 clearly reduced the levels of pro-PDGFA (Fig. 4B), which was accompanied by the appearance of two additional smaller bands (~16 and 34 kDa, Fig. 4B) that are consistent with the predicted monomeric and dimeric forms of active-PDGFA. This experimentally confirmed that PC6 could process pro-PDGFA into its active forms.

Pro-PDGFA is reduced but the active-PDGFA is increased in the endometrial lysates in the mid-secretory compared with the mid-proliferative phase

In women, endometrial PC6 levels are low in the mid-proliferative phase (non-receptive) and significantly increased in the mid-secretory phase (receptive) (Nie et al., 2005; Freyer et al., 2007). We hypothesized that because of PC6 cleavage, more active-PDGFA would be present in the mid-secretory than the mid-proliferative phase. To test this, endometrial tissue lysates from the mid-proliferative and mid-secretory phases were analysed by western blot for the pro- and active-PDGFA forms (Fig. 5). While both forms were detected (Fig. 5A), the pro-PDGFA was reduced but the active-PDGFA was increased in the mid-secretory compared with the mid-proliferative phase (Fig. 5B).

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<th>P-value</th>
<th>Number of unique peptides</th>
<th>Sequence coverage (%)</th>
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Table I Proteins identified as differentially presented in the media of proprotein convertase 6-small interfering RNA (PC6-siRNA) and control HEC1A (human endometrial epithelial) cells.

Total of 16 differently expressed proteins above the threshold (>2-fold change and P-value < 0.01) in the conditioned media of PC6-siRNA compared with control cells were identified based on the Wilcoxon signed-rank test.
Cellular localization of PDGFA in the human endometrium

The localization of the pro- and active-PDGFA in the mid-proliferative (non-receptive) and mid-secretory (receptive) phases was determined by immunohistochemistry (Fig. 6A). The pro-PDGFA showed no distinct localization in any tissues (not shown). In contrast, the active-PDGFA showed a clear difference in both the amount and localization between the two phases. In the mid-proliferative phase, the levels of active-PDGFA were very low and only few cells showed punctuate staining (Fig. 6a and b). In the mid-secretory phase, active-PDGFA was clearly detected on the apical surface of the luminal and glandular epithelium (Fig. 6c and d). These results highlighted the differential localization of the active-PDGFA in the mid-secretory compared with the mid-proliferative phase.

PDGFAA levels in the uterine fluid are significantly higher in the receptivity phase

PDGFAA was previously detected in the uterine fluid (Hannan et al., 2011). We thus determined whether PDGFAA levels were different in the uterine lavage between non-receptive and receptive phases. ELISA analysis of PDGFAA detected a significantly higher concentration ($P = 0.0079$) in the secretory compared with the proliferative phase (Fig. 6B), ranging from 1.59–23.00 pg/ml (mean: 8.96 ± 3.90 pg/ml) in the proliferative phase and 98–297.5 pg/ml (mean: 160.50 ± 35.11 pg/ml) in the secretory phase.

Discussion

In this study, we provide evidence that PC6 mediates the post-translational cleavage of PDGFA in HEC1A cells, and possibly in the...
human endometrium, for receptivity. Using PC6-siRNA cells and proteomics, we identified that pro-PDGFA levels were increased in the conditioned medium of PC6-siRNA versus control cells. We then validated this finding by western blot of media and cell lysates of PC6-siRNA and control cells. We next confirmed by incubating recombinant pro-PDGFA with recombinant PC6 in vitro that PC6 could cleave pro-PDGFA into its active forms. To confirm that the findings derived from HEC1A cells (a carcinoma cell line) are physiologically relevant to the human endometrium, we went further to establish that the pro-PDGFA is also cleaved into active-PDGFA in the human endometrium using western blot and immunohistochemistry. The pro-PDGFA showed no specific localization, but the active-PDGFA was localized to the apical surface of the glandular and luminal epithelium in the mid-secretory phase. Furthermore, the PDGFAA levels in the human uterine fluid were significantly higher in the secretory than the proliferative phase.

Proteomic analysis is a ‘fishing expedition’ and subsequent validation using different approaches are absolutely essential. This study using label-free proteomics identified numerous proteins that show differences in the media of control versus PC6-siRNA cells. We used a 2-fold difference as the cut off to select candidates that were likely different between the two cell types. To further minimize false positives, we bioinformatically analysed the candidate proteins for their potential as PC6 substrates by determining whether they contain potential PC6 cleavage sites. These approaches in combination led us to focus on PDGFA. Our subsequent validation of PC6 regulation of PDGFA used control and PC6-siRNA HEC1A cells (conditioned media and cell lysates) as well as recombinant proteins. To further establish the potential physiological relevance of PC6 regulation of PDGFA, we examined human endometrial tissues by western blot and immunohistochemistry and uterine lavages by ELISA. The relationship between PC6 levels and the detection of different PDGFA forms in different cell compartments is summarized in Fig. 7.

Many functionally important cellular peptides and proteins, including growth factors, neuropeptides and hormones, are initially synthesized as inactive precursors which require post-translational processing to
become biologically active. PCs are known to participate in this important activation. PDGFA is a known precursor protein that requires PC processing to become biologically active (Siegfried et al., 2003). In LoVo cells (colorectal adenocarcinoma), the PC member furin was shown to activate PDGFA (Siegfried et al., 2003; Haouzi et al., 2011).

PC6 is the only PC family member strongly associated with endometrial receptivity (Okada et al., 2005). Our previous studies have established that PC6 is critical for epithelial receptivity (Heng et al., 2011a, b). In the human endometrium, PC6 post-translationally activates cell surface α-integrins (Paule et al., 2012) and a scaffolding protein EBP50 (Heng et al., 2011a).

Figure 5 Western blot of the pro- and active-PDGFA in human endometrial tissue lysates. The pro-PDGFA was reduced but the active-PDGFA was increased in the mid-secretory (Mid-Sec) compared with the mid-proliferative (Mid-Prolif) phase. (A) Representative western blot with E-10 and N-30 antibodies, and (B and C) densitometric analysis of the pro- and active-PDGFA, respectively. Data were normalized to GADPH and are expressed as the mean ± SEM (n = 3–4). Unpaired Student t-test. *P ≤ 0.05.

Figure 6 (A) Immunolocalization of the active-PDGFA in the human endometrium. Representative images with N-30 antibody for the Mid-Prolif (a and b; n = 6) and Mid-Sec (c and d; n = 6) phases are shown. Scale bars in all images represent 20 μm. (B) ELISA detection of PDGFA in the uterine lavage collected from the proliferative (Prolif, n = 5) and secretory (Sec, n = 5) phases of the menstrual cycle. Data are expressed as mean ± SEM. **P < 0.01.

Figure 7 A schematic diagram showing the relationship between PC6 levels and the detection of different PDGFA forms in cell lysates and media. When PC6 levels are low, pro-PDGFA cleavage is prevented and the pro-form is detected predominantly in cell lysates as well as the conditioned media. When PC6 levels are high, pro-PDGFA is cleaved into the active form within the cell and released into the conditioned media.
In this study, we show that PC6 regulates the cleavage of growth factor PDGFA in HEC1A cells and in human endometrial epithelium during preparation for receptivity.

We found that the pro-PDGFA accumulated in the HEC1A cells when PC6 was knocked down. This is in agreement with previous reports of pro-PDGFA accumulation following the addition of PC inhibitors in HK293 cells that were transfected with a construct for pro-PDGFA-A (Mermola et al., 1990). We further established a link between PC6 concentrations and PDGFA activation in the human endometrium. PC6 levels are low in the non-receptive phase and significantly increased in the receptive phase (Heng et al., 2011a, b). The concentrations of the active-PCG6A are also higher in the receptive than the non-receptive phase. We detected very little active-PDGFA in the endometrial sections in the non-receptive phase when PC6 levels are low, but in the receptive phase when PC6 levels are high, the active-PDGFA levels were increased and localized to the apical surface of the glandular and luminal epithelium. In addition, the levels of PDGFAA in the uterine lavage were significantly increased in the receptive phase. These data are consistent with pro-PDGFA cleavage/activation of pro-PDGFA in the human endometrium in the receptive phase. This is also consistent with the previous report that proteolytic cleavage of PDGFA is necessary for cell surface localization and efficient release of active-PDGFA from the cell (LaRochelle et al., 1991).

In summary, our studies demonstrate that endometrial PC6 is an important regulatory factor in the post-translational conversion/activation of PDGFA for receptivity. Failure of this conversion would decrease the binding of PDGFA to its receptors for downstream signalling that may be important for endometrial receptivity. This study also suggests that the active-PDGFA in uterine fluid may serve as potential biomarker for endometrial receptivity.

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**Authors’ roles**

S.P.: HEC1A cell culture and western blot analysis, in vitro PC6 cleavage experiment, human endometrial tissue and lavage studies, and manuscript drafting. T.N. and A.I.W.: proteomic analysis. B.V. and L.J.F.R.: critical discussion and manuscript review. G.N.: overall study design, data analysis and manuscript writing.

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**Conflict of interest**

B.V. has shares in Monash IVF; the other authors declare no conflict of interest.

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