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The presence and regulation of connective tissue growth factor in the human endometrium

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BACKGROUND: The human endometrium efficiently repairs each month after menstruation. The mechanisms involved in this repair process remain undefined. Aberrations in endometrial repair may lead to the common disorder of heavy menstrual bleeding. We hypothesized that connective tissue growth factor (CTGF) is increased at the time of endometrial repair post-menses and that this increase is regulated by prostaglandins (PGs) and hypoxic conditions present during menstruation.

METHODS AND RESULTS: Examination of 41 endometrial biopsies from 5 stages of the menstrual cycle revealed maximal CTGF mRNA expression (using quantitative RT–PCR) at menstruation and peak protein levels during the proliferative phase. CTGF was immunolocalized to epithelial and stromal cells, with intense staining of occasional stromal cells during the proliferative phase. Dual immunohistochemistry identified these cells as macrophages. Treatment of endometrial epithelial cells with 100 nM PGE_2, $PGF_{2\alpha}$ or hypoxia (0.5% O_2) revealed a significant increase in CTGF mRNA expression ($P < 0.01$ for all, versus vehicle control). Cells treated simultaneously with PGE_2 and hypoxia revealed a synergistic increase in CTGF expression ($P < 0.05$ versus PGE_2 or hypoxia alone) and maximal secreted CTGF protein levels ($P < 0.05$ versus control).

CONCLUSIONS: CTGF is increased in the human endometrium at the time of endometrial repair post-menses. The increase in CTGF may be mediated by PG production and the transient hypoxic episode observed in the endometrium at menstruation.

Key words: menstruation / repair / prostaglandins / hypoxia / macrophage

Introduction

The human endometrium is an excellent physiological model of cyclical tissue injury and repair. If pregnancy does not occur, the corpus luteum regresses in the late secretory phase and progesterone levels decline. This withdrawal of progesterone triggers an inflammatory response in the endometrium, which culminates in the menstrual shedding of the functional endometrial layer (Critchley *et al.*, 2001; King and Critchley, 2010). Subsequent repair is very efficient, occurring without scarring or loss of function. However, the precise mechanisms involved remain poorly understood. Observational studies have shown that endometrial repair begins on Day 2, during active bleeding, and full coverage of the lumen is usually achieved by Day 6 (Ludwig and Spornitz, 1991). In addition, the endometrium is shed in a piecemeal fashion at menstruation with simultaneous repair occurring in adjacent areas (Garry *et al.*, 2009). These studies highlight that factors involved in the initiation of endometrial repair 'post-menses' must be present at the time of menses itself.

Connective tissue growth factor (CTGF) is a multifunctional growth factor that is expressed at high levels during wound repair and at sites of connective tissue formation (Igarashi *et al.*, 1993). Its biological effects include chemotaxis, differentiation, extracellular matrix (ECM) production, angiogenesis, tumour growth, wound healing and fibrosis (Igarashi *et al.*, 1993; Frazier *et al.*, 1996; Oemar *et al.*, 1997; Hishikawa *et al.*, 2000; Shimo *et al.*, 2001; Ivkovic *et al.*, 2003). It is, therefore, an attractive candidate for endometrial repair. CTGF protein and mRNA have previously been detected in the human endometrium by immunohistochemistry and northern blot analysis, respectively (Uzumcu *et al.*, 2000). CTGF was localized to epithelial and endothelial cells in proliferative and secretory phase biopsies. Whereas, in contrast, endometrial stromal cells were only stained after decidualization in the late secretory phase (Uzumcu *et al.*, 2000). Unfortunately, no menstrual phase biopsies were included in this study, but the presence of CTGF in the normal cycling endometrium suggests that it may facilitate tissue repair and remodelling after menses.

Premenstrual progesterone withdrawal increases levels of cyclo-oxygenase (COX) 2 enzyme in the endometrium (Critchley *et al.*, 1999), which stimulates the synthesis of endometrial prostaglandins (PGs), including prostaglandin E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$). $PGF_{2\alpha}$ is emerging as a potential key player in the initiation of expression of endometrial repair factors. PGs have been demonstrated to induce a host of endometrial angiogenic factors including vascular endothelial growth factor, adrenomedullin, fibroblast growth factor-2 and interleukin-8 (Keightley *et al.*, 2010; Maybin *et al.*, 2011a,b,c). Functionally, $PGF_{2\alpha}$ has been shown to increase endometrial epithelial cell proliferation in culture (Milne and Jabbour, 2003) and is known to induce vasoconstriction of endometrial spiral arterioles (Baird *et al.*, 1996). There is evidence that this vasoconstriction results in a transient, local hypoxic insult in the uppermost endometrial layer at menstruation (Critchley *et al.*, 2006; Fan *et al.*, 2008).

We hypothesized that CTGF is increased at the time of endometrial repair and regeneration and that this putative repair factor is regulated by PGs and hypoxia.

Materials and Methods

Human endometrial tissue collection

Endometrial biopsies ($n = 41$) were collected with a suction curette (Pipelle, Laboratorie CCD, Paris, France) from women (median age: 42 years, range: 22–50) attending gynaecological out-patient departments across National Health Service Lothian. All women reported regular menstrual cycles (21–35 days) and no exogenous hormone exposure for 3 months prior to biopsy. Women with large fibroids (>3 cm) or endometriosis were excluded. Tissue was divided and (i) placed in RNA later, RNA stabilization solution [Ambion (Europe) Ltd., Warrington, UK], (ii) fixed in neutral-buffered formalin for wax embedding and (iii) placed in phosphate-buffered saline for *in vitro* culture. Biopsies were consistent for (i) histological dating using criteria of Noyes *et al.* (1950), (ii) reported last menstrual period and (iii) serum progesterone and estradiol concentrations at time of biopsy (Table I). Written consent was obtained from participants and ethical approval granted from Lothian Research Ethics Committee.

Immunohistochemistry

Three micrometres of paraffin sections was dewaxed and rehydrated. Antigen retrieval was undertaken by microwaving sections in a pH 6 antigen-retrieval buffer (Vector Laboratories, Peterborough, UK). Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Sections were sequentially incubated in avidin and biotin (Vector Laboratories) and protein block (Dako, Cambridge, UK). Goat polyclonal CTGF antibody (Santa Cruz Biotechnology, CA, USA) was applied overnight at 4°C. Negative controls were incubated with primary antibody pre-absorbed in antigen (CTGF peptide, Santa Cruz). Biotinylated horse anti-goat secondary antibody was used at 1:200. Avidin–biotin–peroxidase complex (ABC-Elite, Vector laboratories) was applied for 30 min and liquid diaminobenzidine kit (Zymed Laboratories, San Francisco, CA, USA) used for detection. The reaction was stopped with distilled water and the sections were counterstained with haematoxylin, dehydrated and mounted with Pertex (Cellpath plc, Hemel Hempstead, UK).

Dual staining immunohistochemistry demonstrating co-localization of CD68 and CTGF

Endometrial sections underwent dewaxing, rehydration, antigen retrieval and treatment with 3% hydrogen peroxidase as above. Normal donkey serum was used as a protein block and the sections were incubated with mouse monoclonal CD68 (a pan-macrophage antigen) antibody (Dako, Glostrup, Denmark) at a 1 in 1000 dilution overnight at 4°C. Donkey anti-mouse peroxidase secondary antibody (Abcam, Cambridge, UK) at a 1:750 dilution was applied for 30 min followed by incubation with TSA™ fluorescein tyramide system (Perkin Elmer, Waltham, MA, USA) for 10 min. The sections were incubated with normal donkey serum for 10 min followed by goat polyclonal CTGF antibody (Santa Cruz Biotechnology) at a 1 in 100 dilution overnight at 4°C. Alexa 546 donkey anti-goat secondary antibody (Invitrogen, Paisley, UK) was applied for 1 h, followed by a 4′,6-diamidino-2-phenylindole stain (Sigma, Dorset, UK) for 10 min. Sections were then mounted with permaflour (Thermo Scientific, Waltham, MA, USA) and analysed with a Zeiss LSM710 confocal microscope system.

Semi-quantitative histoscoring of endometrial CTGF expression

Localization and intensity of immunostaining was evaluated blindly by two independent observers using a semi-quantitative scoring system (Aasmundstad *et al.*, 1992). The intensity of staining was graded with a three-point scale (0 = no staining, 1 = mild staining and 2 = strong staining). This was applied to the glands and stromal cells ($n = 41$), as well as the surface epithelium where visualized ($n = 36$). The percentage of tissue in each intensity scale was recorded. A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining.

Table I Human endometrial biopsies used in this study.

Stage of cycle	Number of biopsies	Mean serum estradiol levels, pmol/l (range)	Mean serum progesterone, nmol/l (range)
Menstrual	8	192.25 (55–514)	3.71* (1.24–10.59)
Proliferative	11	441.18 (79–1105)	2.81* (0.97–7.10)
Early secretory	7	497.50 (289–841)	59.60 (23.2–112.91)
Mid-secretory	8	638.00 (242–1949)	64.30 (25.47–114.53)
Late secretory	7	318.22 (59.09–819)	8.22* (1.06–16.95)

*Significantly different from early and mid-secretory levels, $P < 0.05$.

Culture of endometrial cells

Human Ishikawa endometrial adenocarcinoma cells (European collection of cell culture, Centre for Applied Microbiology, Wiltshire, UK) were stably transfected with the $PGF_{2\alpha}$ receptor (FPS cells) or a PGE_2 receptor (EP2S cells) as previously described (Sales *et al.*, 2004). To determine the effect of $PGE_2/F_{2\alpha}$ and/or hypoxic conditions on CTGF expression, $\sim 4 \times 10^5$ FPS or EP2S cells were seeded in 6-well plates in Dulbecco's modified eagle's medium F-12 supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 200 μg/ml G418. The following day, cells were incubated in serum-free culture medium containing 8.4 μM indomethacin (COX enzyme inhibitor) for at least 16 h. Cells were then treated with vehicle or 100 nM $PGE_2/F_{2\alpha}$ and placed in normoxia (21% O_2, 5% CO_2, 37°C) or a sealed hypoxic chamber (0.5% O_2, 5% CO_2, 37°C) for 2, 4, 6, 8 or 24 h.

Culture of endometrial tissue explants

Endometrial biopsies (proliferative $n = 3$, secretory $n = 3$) were divided into four explants of equal size and incubated on raised platforms in 24-well plates, just covered with serum-free RPMI 1640 medium and 8.4 μM indomethacin for 16 h. One explant from each biopsy was then treated with vehicle in normoxia, one with 100 nM $PGF_{2\alpha}$ in normoxia, one with 100 mM PGE_2 in normoxia and one with vehicle in hypoxia for 24 h.

Quantitative RT–PCR

Expression of CTGF in endometrial tissue and cells was determined by quantitative RT–PCR (QRT–PCR, Taqman) analysis. Total RNA from cells and endometrial biopsies was extracted using the RNeasey Mini Kit (Qiagen Ltd, Sussex, UK) according to manufacturer's instructions. DNA contamination of samples was removed by DNA digestion during RNA purification. RNA samples were reverse transcribed using $MgCl_2$ (5.5 mM), deoxynucleotide triphosphates (0.5 mM each), random hexamers (2.5 μM), ribonuclease inhibitor (0.4 U/μl) and multiscribe reverse transcriptase (1.25 U/μl; all from PE Biosystems, Warrington, UK). Total RNA of 200 ng was added. A tube with no reverse transcriptase and a further tube with water were included as controls. To measure cDNA levels, a reaction mix was prepared containing Taqman buffer (5.5 mM $MgCl_2$, 200 μM dATP 200 μM dCTP, 200 μM dGTP and 400 μM deoxyuridine triphosphate), ribosomal 18S primers/probe (Applied Biosystems, Warrington, UK) and specific forward and reverse primers and probes: CTGF forward primer 5′-TGCACCGCCAAAGATGGT-3′, reverse primer 5′-GGCACGTGCACTGGTACTTG-3′, probe 5′-CTCCCTGCATCTTCGGTGGTACGGT-3′ was added for each PCR reaction. Negative controls (water instead of cDNA) were included in each run. PCR was carried out using an ABI Prism 7900 (Applied Biosystems). Samples were analysed in triplicate using Sequence Detector version 2.3 (PE Biosystems), using the comparative threshold method. Expression of target mRNA was normalized to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. There was no significant change in 18S rRNA expression between normoxic and hypoxic conditions.

CTGF protein quantification

CTGF secreted protein concentrations were measured using a human CTGF ELISA development kit (PeproTech, London, UK) according to manufacturer's instructions. Colour development was measured on an enzyme-linked immunosorbent assay plate reader at 405 nm. The minimum detectable concentration of CTGF was 63 pg/ml. There was no significant cross-reactivity for human bone morphogenetic protein-4, CTGF-L/WNT inducible signalling pathway (WISP)-2, cystine rich protein-61, insulin-like growth factor (IGF)-I, IGF-II, IGF-BP1, nephroblastoma overexpressed gene, transforming growth factor-β, WISP-1 or WISP-3.

Statistics

For endometrium from across the menstrual cycle, mRNA results were expressed as the quantity relative to a comparator sample of RNA from the liver. Significant differences in mRNA and protein were determined using Kruskal–Wallis non-parametric test with Dunn's multiple comparison post-test (Prism, version 4.02, GraphPad Software, Inc., San Diego, CA, USA). For cell culture, mRNA results are expressed as fold increase, where relative expression of mRNA after treatment was divided by the relative expression after vehicle treatment. Data are presented as mean ± SEM and significant differences among raw data determined using one-way analysis of variance with Tukey's multiple comparison test. A value of $P < 0.05$ was considered significant.

Results

Endometrial CTGF mRNA expression

Endometrial CTGF mRNA expression varied significantly across the menstrual cycle ($P = 0.0032$; Fig. 1). Maximal CTGF mRNA expression was present in endometrium from the menstrual phase, with levels significantly greater than those found during the early ($P < 0.01$), mid- ($P < 0.05$) and late ($P < 0.05$) secretory phases.

Endometrial CTGF protein localization

CTGF was immunolocalized to the cytoplasm of surface epithelial, glandular epithelial, stromal and vascular endothelial cells or

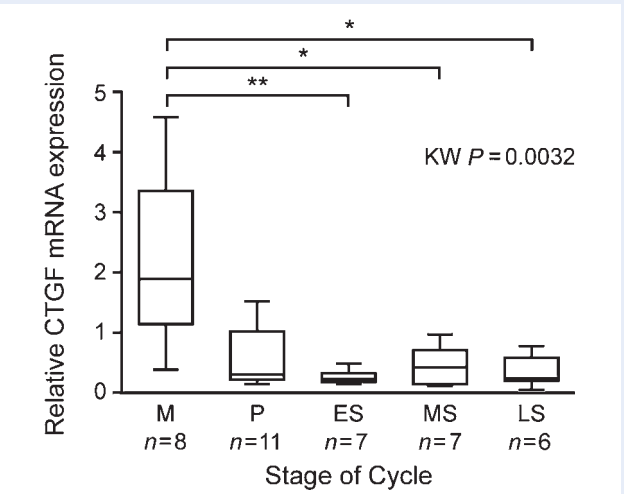

Figure 1 CTGF mRNA expression in human endometrial tissue from across the menstrual cycle. Values of CTGF are presented as relative to the 18S ribosomal RNA internal standard and to a sample of RNA from the liver as a comparator. M, menstrual; P, proliferative; ES, early secretory; MS, mid-secretory; LS, late secretory; KW, Kruskal–Wallis statistical test (*$P < 0.05$, **$P < 0.01$). Box and whisker plots: each box represents the 25th and 75th percentiles and the whiskers are the 10th and 90th percentiles. Horizontal lines represent the median.

endometrium from the menstrual and proliferative phases (Fig. 2A–D). There was an obvious reduction in staining intensity during the secretory phase (Fig. 2E and F). Intense staining of occasional cells in the stromal compartment of proliferative endometrium was observed (Fig. 2H). These cells were usually found in close proximity to the endometrial vasculature. Semi-quantitative scoring of immunohistochemical staining revealed significantly greater staining of the glandular epithelial, surface epithelial and stromal cells in proliferative phase endometrium when compared with mid-secretory endometrium ($P < 0.001$, $P < 0.001$, $P < 0.01$ respectively; Fig. 3).

Stromal CTGF was immunolocalized to macrophage cells

Proliferative endometrial tissue was subjected to dual staining immunohistochemistry for CTGF and CD68 (a pan-macrophage antigen). This revealed that the occasional stromal cells that were positively stained for CTGF also expressed CD68 (Fig. 4). Glandular epithelial cells were positively stained for CTGF but did not reveal dual staining for CD68.

CTGF expression was regulated by PGs and hypoxia

A time course experiment in EP2S cells revealed a significant increase in CTGF mRNA expression on treatment with 100 nM PGE_2 compared with vehicle at 4 and 8 h ($P < 0.01$) (Fig. 5A). Hypoxic conditions (0.5% O_2) also significantly increased CTGF mRNA expression in EP2S cells at 2 h versus normoxic controls ($P < 0.001$; Fig. 5B). Interestingly, in prolonged hypoxic conditions this up-regulation did not take place. When cells were treated with both PGE_2 and hypoxia for 2 h, there was a synergistic increase in CTGF mRNA expression that was significantly greater than that seen with either treatment alone ($P < 0.05$; Fig. 5C). As maximal CTGF mRNA expression occurred between 2 and 4 h, the amount of CTGF protein secreted into culture supernatants was assessed by ELISA at a 6 h time point. CTGF secreted protein was below the level of detection of the ELISA in vehicle-treated cells but was significantly increased when EP2S cells were co-treated with PGE_2 and hypoxia ($P < 0.05$; Fig. 5D).

Treatment of FPS cells with 100 nM $PGF_{2\alpha}$ for 6 h resulted in significant up-regulation of CTGF mRNA expression versus vehicle-treated cells ($P < 0.001$; Fig. 5E). As in EP2S cells, hypoxic conditions did not significantly increase CTGF mRNA expression at 6 h in FPS cells (Fig. 5E). Secreted protein levels in culture supernatants at 24 h demonstrated significant increases in CTGF protein over controls after treatment with $PGF_{2\alpha}$ ($P < 0.001$) (Fig. 5F).

Treatment of endometrial explants from the proliferative phase showed a modest increase in CTGF mRNA expression when explants were placed in hypoxic conditions ($P < 0.05$; Fig. 5G). Explants from the secretory phase displayed a more marked increase in CTGF mRNA expression in hypoxic conditions ($P < 0.001$; Fig. 5H). No significant increase in CTGF mRNA was observed when explants were treated with PGs, regardless of cycle stage.

Discussion

These data demonstrate maximal expression of CTGF in the human endometrium during the menstrual and proliferative phases; a time consistent with endometrial repair and regeneration. In addition, CTGF protein is localized to glandular epithelial cells and macrophages in the stromal cell compartment. Furthermore, this paper describes significant increases in CTGF mRNA and secreted protein in endometrial cells treated with PGs and/or hypoxia. These data provide a potential mechanism for the up-regulation of CTGF during the time of endometrial repair associated with menstruation.

The location and timing of endometrial repair

The data presented herein were generated from examination of endometrial biopsies collected with an endometrial sampler. The majority of endometrium collected with this method is from the functional layer. As this layer is sloughed off during menses, it has hitherto been assumed that endometrial repair was governed by factors present in the basal layer. An important recent study of menstrual endometrium from the superficial layer has revealed an increase in expression of genes associated with ECM biosynthesis in stromal cells from this upper layer when compared with those from the basal layer (Gaide Chevronnay *et al*., 2009). This suggests that fragments of the functional layer of endometrium do make an active contribution to the endometrial repair process. Further, recent laser capture microdissection techniques have identified that both the stromal and epithelial cell compartments have an active role in this repair process (Gaide Chevronnay *et al*., 2010).

Hysteroscopic, histological and scanning electron microscopy studies have documented the time course of endometrial repair. The endometrium is shed in a piecemeal fashion and repair commences in delineated areas while shedding occurs simultaneously in adjacent areas (Garry *et al*., 2009). Therefore, examination of endometrial repair factors during the menstrual phase is essential when examining the process of repair in the context of menstruation.

CTGF and endometrial repair

CTGF is a member of the CCN family of growth regulators (Brigstock, 2002) and has a putative role in endometrial repair. Data herein demonstrate maximal CTGF mRNA expression in menstrual phase endometrium. CTGF protein showed intensely positive immunohistochemical staining during the proliferative phase, which was significantly elevated in glandular epithelial, surface epithelial and stromal cells when compared with the mid-secretory phase. This is the first description of CTGF mRNA expression and protein staining intensity in human endometrium from all stages of the menstrual cycle, including at menstruation. A previous study immunolocalized human endometrial CTGF to epithelial, stromal and endothelial cells but did not examine endometrium from the menstrual phase (Uzumcu *et al*., 2000). In contrast to findings described herein, Uzumcu *et al* (2000) found minimal staining of stromal cells in proliferative tissue and maintenance of epithelial staining throughout the secretory phase. These differences may be explained by varying antibody specificities or by the classification of endometrial tissue studied. We have carefully staged the tissues used in this study based on reported day of cycle, serum ovarian hormone levels on the day of biopsy and histological dating. Other members of the CCN family also show significant variation in human endometrial expression across the cycle. The pro-angiogenic factor cysteine rich protein 61 (CYR61) was found

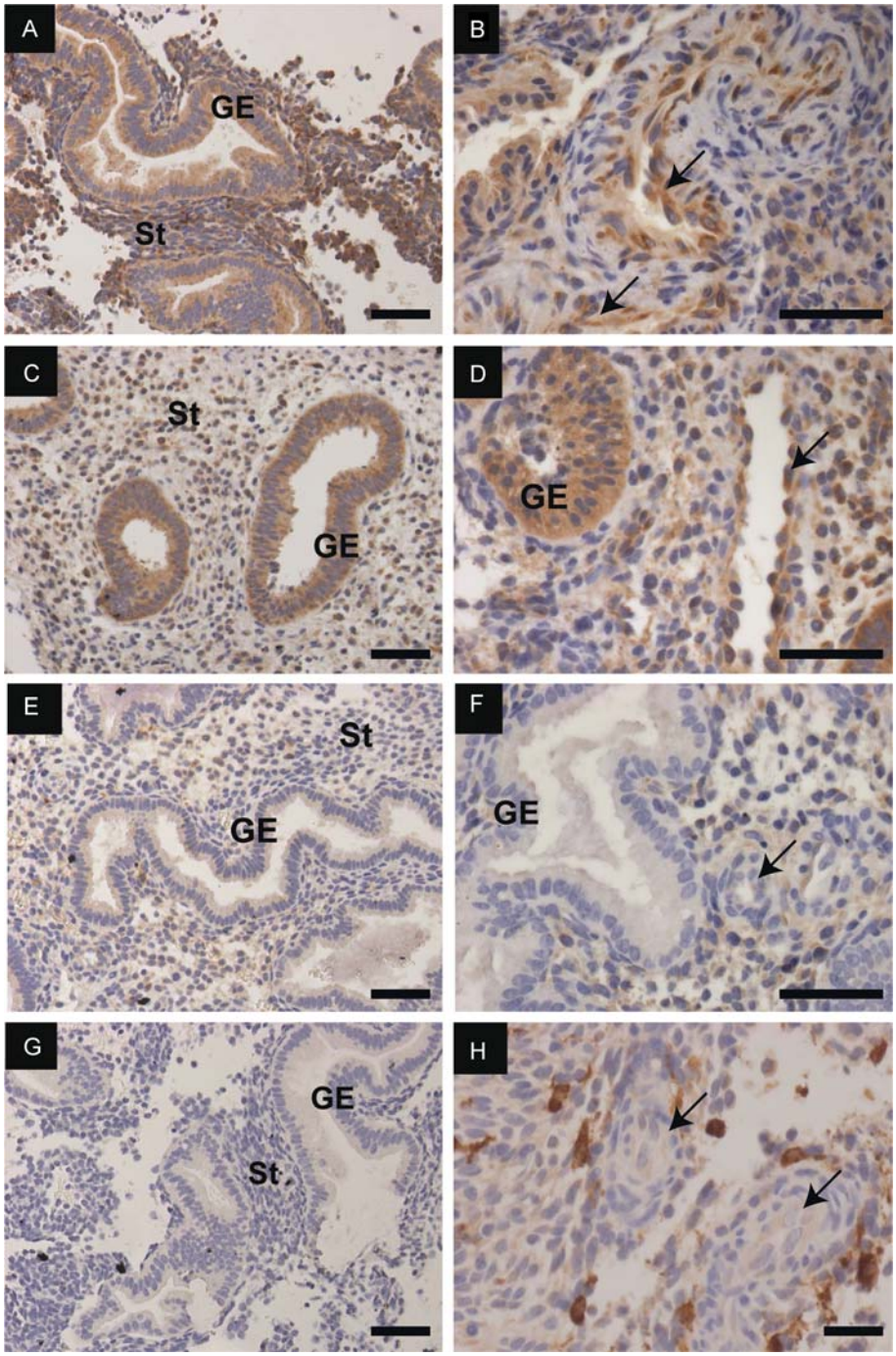

Figure 2 CTGF immunohistochemical staining in human endometrium from across the menstrual cycle: (**A** and **B**) menstrual phase endometrium, (**C** and **D**) proliferative phase endometrium, (**E** and **F**) mid-secretory phase endometrium, (**G**) negative control menstrual endometrium, incubated with the primary antibody pre-absorbed with blocking peptide and (**H**) intense staining of occasional cells (arrows) in the stromal cell compartment of proliferative endometrium. Scale bar = 50 μm. Arrows, endothelial cells; GE, glandular epithelial cells; St, stromal compartment.

to be significantly elevated during menses (Gashaw *et al.*, 2008) and authors proposed it contributes to repair, growth and maturation of the endometrial vasculature after menstruation.

The mechanism by which CTGF exerts its effects remains undefined at a molecular level, therefore examination of receptor expression for this factor was not possible. CYR61 (a member of the CNN family) has been shown to bind to integrin $\alpha_v\beta_3$ to mediate cell adhesion and migration (Kireeva *et al.*, 1998) and CTGF may elicit its biological effects in a similar manner. Alternatively, it has been proposed that the CCN family may not signal exclusively via cell surface receptors but rather are a component of the ECM, bridging the functional gap between structural and active molecules (Lau and Lam, 1999).

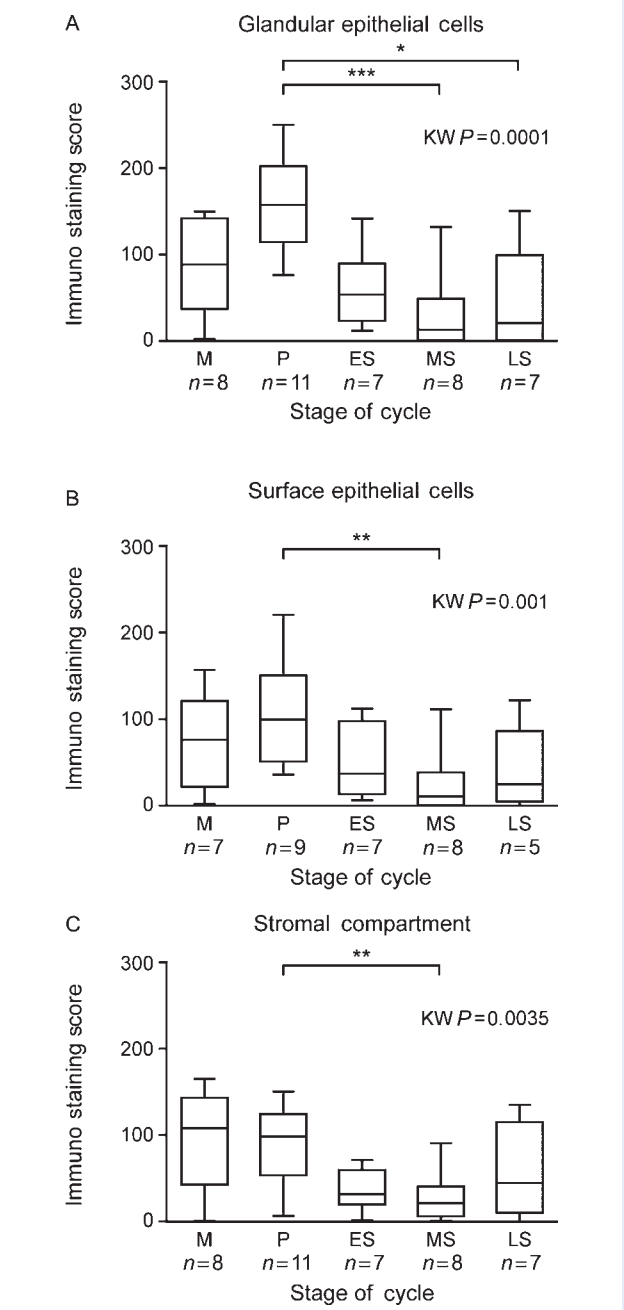

Figure 3 Semi-quantitative scoring of human endometrial CTGF immunohistochemical staining across the menstrual cycle. (**A**) Glandular epithelial cells, (**B**) surface epithelial cells and (**C**) the stromal cell compartment. KW, Kruskal–Wallis statistical test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Other components of the ECM are known to have a fundamental role in the repair process and have a similar endometrial pattern of expression to that of CTGF. Fibronectin (FN) is a large glycoprotein that interacts with specific integrins to enhance cell adhesion and migration during wound repair (Kim *et al*., 1992). Utilizing the ovariectomized rhesus macaque model, Cao *et al*. (2007) demonstrated a 50-fold increase in FN expression from the secretory to the menstrual phase alongside a 17-fold increase in its receptor, integrin-β1. Real-time PCR, *in situ* hybridization and immunohistochemistry confirmed these findings and localized the increase in FN expression to the uppermost endometrial glands and luminal epithelium. Significant increases in adhesive molecules, such as CTGF and FN, in the functional layer of the endometrium at this time implicate involvement in surface healing post-menstruation.

Interestingly, we observed intense CTGF immunostaining of occasional stromal cells during the proliferative phase. Dual staining immunohistochemistry identified these cells as macrophages. Macrophages have a fundamental role in the inflammatory response and are often found at sites of intense tissue remodelling. They have been detected in the endometrium throughout the menstrual cycle (Bonatz *et al*., 1992) with a significant influx during the late secretory phase (Kamat and Isaacson, 1987). The importance of endometrial leukocytes in post-menstrual repair has been demonstrated in the mouse model of menstruation. Neutrophil depletion using the antibody RB6-8C5 markedly delayed endometrial repair when examined 48 h after progesterone withdrawal versus control animals (Kaitu'u-Lino *et al*., 2007). The role of macrophages during endometrial repair is still to be fully defined but macrophage depletion in the mouse model of endometriosis suggests a crucial role in growth and vascularization (Bacci *et al*., 2009). Detection of CTGF within endometrial macrophages is thought to be of an endocytotic nature from the surrounding environment where it potentially acts as a chemoattractant to peripheral mononuclear cells (Cicha *et al*., 2005). CTGF may be involved in the influx of macrophages to the endometrium during the latter stages of the menstrual cycle. There is recent evidence suggesting communication between macrophages and stromal cells in the human endometrium (Eyster *et al*., 2010). Endometrial stromal cells co-cultured with macrophage cell supernatant demonstrated significantly increased expression of CTGF over those cultured with vehicle. Moreover, this response was found to be abrogated in the presence of estradiol and progesterone. Hence, endometrial macrophages may contribute to the significant increase in stromal cell expression of CTGF at the time of menstruation, when ovarian hormone levels are low.

Hypoxia and the induction of repair

Herein we have demonstrated a significant increase in CTGF mRNA after exposure of endometrial epithelial cells and endometrial tissue explants to hypoxic conditions. The timing of increased CTGF in response to hypoxia was different in endometrial cells and tissue explants, with cells demonstrating a rapid response and tissue maintaining increased CTGF expression at 24 h. This may reflect the heterogeneous cell population that constitutes endometrial tissue. Tissue explants allow maintenance of cell–cell communication *in vitro*, mimicking the *in vivo* scenario more accurately than single cell culture.

It is postulated that a period of transient hypoxia occurs *in vivo* in the uppermost endometrial zones as spiral arterioles constrict following progesterone withdrawal. An early study in the rhesus monkey (Markee, 1940) revealed intense endometrial vasoconstriction, tissue necrosis and menstruation following progesterone withdrawal. More recently, the use of pimonidazole (hydroxyprobe®), a marker of oxygen partial pressures <10 mmHg, in the mouse model identified the presence of hypoxia during simulated menstruation (Fan *et al*.,

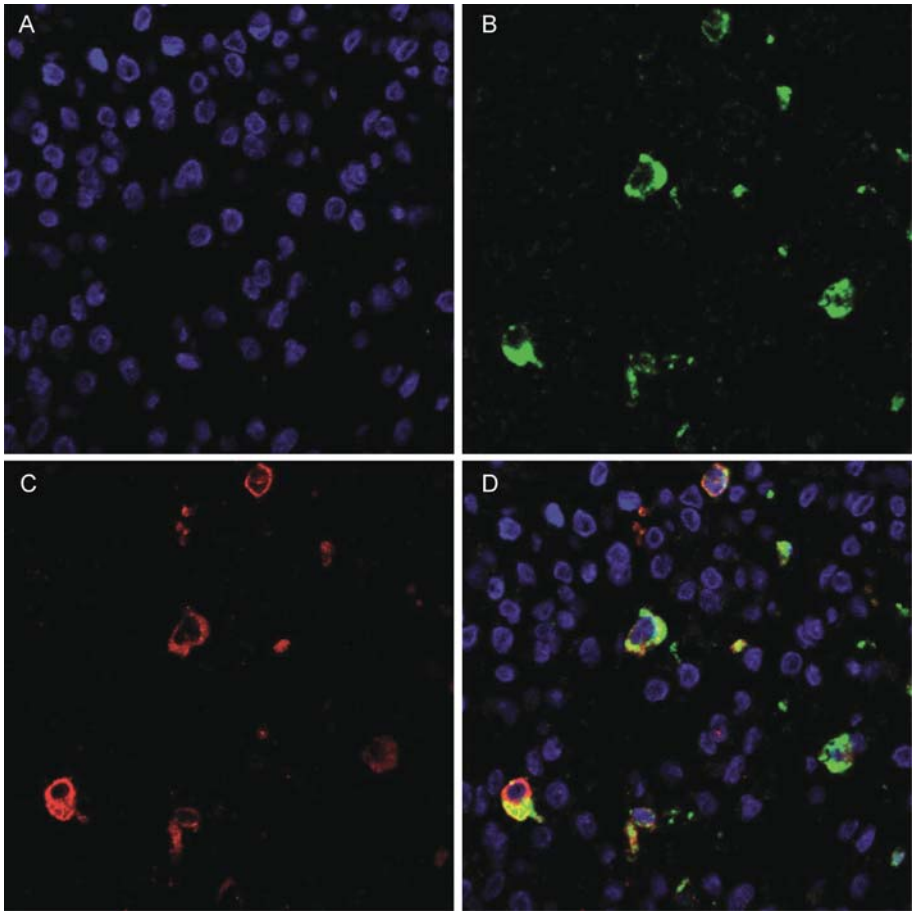

Figure 4 Dual staining immunolocalization of CTGF and CD68 in endometrium from the proliferative phase: (**A**) nuclei staining in blue, (**B**) CD68 staining in green, (**C**) CTGF staining in red and (**D**) dual staining for CD68 (green) and CTGF (red) showing co-localization of CTGF in macrophage cells.

2008). In contrast, negligible levels of pimonidazole were observed by Day 5 after progesterone withdrawal. Furthermore, markers of tissue hypoxia (CAIX and hypoxia inducible factor-1α) have been detected immunohistochemically in the human endometrium during menstruation, with a distinct reduction in staining of both markers after cycle Day 5 (Critchley *et al.*, 2006; Punyadeera *et al.*, 2006).

Prostaglandins and the induction of repair

We also demonstrate significant increases in CTGF mRNA and protein expression in endometrial cells after incubation with PGE_2 and $PGF_{2\alpha}$. These results contrast with findings in rat fibroblast cells and human airway smooth muscle cells, where PGE_2 treatment decreased CTGF mRNA expression (Yu *et al.*, 2002; Burgess *et al.*, 2006); highlighting the cell and tissue-specific nature of CTGF induction. We have demonstrated a synergistic increase in CTGF mRNA expression in endometrial cells treated with PGE_2 and hypoxia simultaneously. Both PGE_2 and $PGF_{2\alpha}$ have previously been shown to increase endometrial epithelial cell CYR61 mRNA expression (Gashaw *et al.*, 2008). Furthermore, a synergistic increase with both hypoxia and PGE_2 was also demonstrated for this other member of the CCN family. Of note, CTGF mRNA was not increased in response to $PGE_2/F_{2\alpha}$ in endometrial tissue explants examined herein. This may be explained by a lack of FP receptors (the $PGF_{2\alpha}$ receptor) in human endometrial stromal cells (Milne and Jabbour, 2003), which may mask any epithelial increase in CTGF mRNA in whole tissue explants. Alternatively, the response of endometrial tissue to PG exposure *in vitro* may occur at a different time point. Owing to the limitations of human tissue availability, time-course experiments could not be performed.

As hypoxic conditions and increased PG synthesis have been demonstrated in the premenstrual endometrium following progesterone withdrawal, these factors may have an important role in the induction of CTGF. Their independent and synergistic actions may provide a robust mechanism for the stimulation of endometrial repair. Transforming growth factor (TGF)-β is well established as a transcriptional regulator of CTGF in other cell types (Harlow *et al.*, 2002; Higgins *et al.*, 2004). The role of TGF-β and other inflammatory mediators in endometrial CTGF expression remains to be determined.

Conclusions

This paper describes maximal CTGF levels in the menstrual and proliferative endometrium; a time consistent with endometrial repair. We

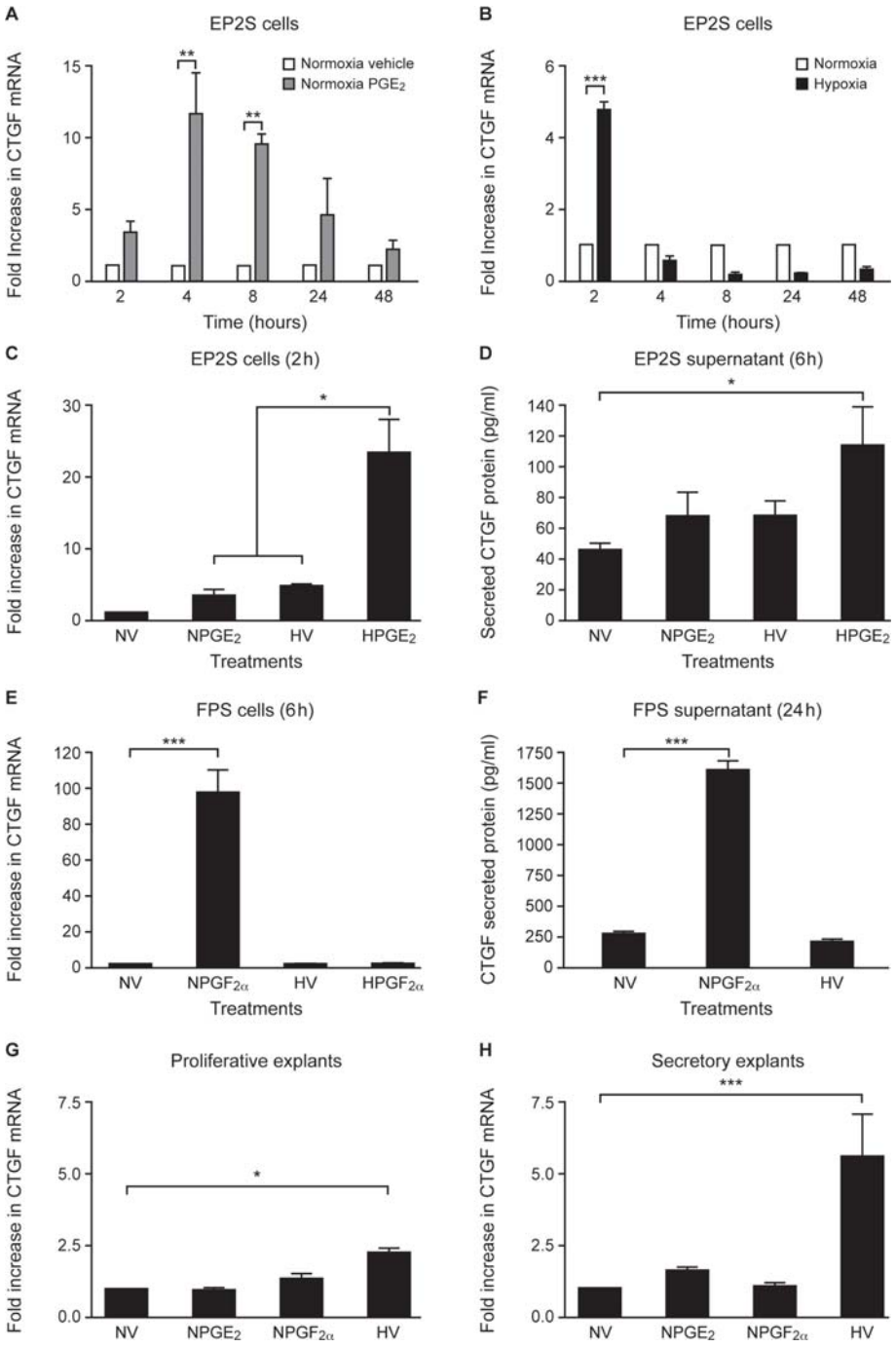

Figure 5 The regulation of CTGF by hypoxia and prostglandins. (**A**) CTGF mRNA expression in EP2S cells treated with vehicle or 100 nM PGE_2 for up to 48 h in normoxic conditions ($n = 3$). (**B**) CTGF mRNA expression in EP2S cells cultured in normoxic and hypoxic conditions for up to 48h ($n = 3$). (**C**) CTGF mRNA expression in EP2S cells treated with 100 nM PGE_2 and/or hypoxia for 2 h ($n = 3$). (**D**) Secreted CTGF protein levels in EP2S cell culture supernatant at 6 h ($n = 3$). (**E**) CTGF mRNA expression in FPS cells treated with 100 nM PGE_2 and/or hypoxia for 6 h ($n = 3$). (**F**) Secreted CTGF protein levels in FPS cell culture supernatant at 24 h ($n = 3$). (**G**) CTGF mRNA in endometrial tissue explants from the proliferative phase treated with vehicle, 100 nM PGE_2, 100 nM $PGF_{2\alpha}$ or hypoxia ($n = 3$). (**H**) CTGF mRNA in endometrial tissue explants from the secretory phase treated with vehicle, $PGF_{2\alpha}$, PGE_2 or hypoxia ($n = 3$). N, normoxia (21% O_2), H, hypoxia (0.5% O_2); V, ethanol vehicle; PG, prostaglandin (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$). Fold changes are relative to findings in vehicle-treated controls in normoxic conditions.

describe the induction of CTGF by PGE_2, $PGF_{2\alpha}$ and hypoxic conditions and suggest these factors have a role in the perimenstrual endometrium to up-regulate CTGF expression for endometrial repair. Further studies are required to determine if aberrations in perimenstrual PG production or vasoconstriction lead to decreased CTGF levels and delayed repair, resulting in the clinical complaint of heavy or prolonged menstrual bleeding.

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Authors' roles

J.A.M. contributed to conception and design, acquired data, analysed and interpreted data and drafted the article. J.B. and U.T. acquired data, performed analysis and provided critical revisions of the manuscript. N.H., H.N.J. and H.O.D.C. contributed to conception and design and provided critical revision of the intellectual content of the manuscript. All authors provided final approval of the version to be published.

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Conflict of interest

None declared.

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