



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding

Citation for published version:

Maybin, J, Boswell, L, Young, VJ, Duncan, W & Critchley, H 2017, 'Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding', *J Clin Endocrinol Metab*, vol. 102, no. 4, pp. 1299-1308. <https://doi.org/10.1210/jc.2016-3437>

Digital Object Identifier (DOI):

[10.1210/jc.2016-3437](https://doi.org/10.1210/jc.2016-3437)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

J Clin Endocrinol Metab

Publisher Rights Statement:

Author's final peer-reviewed manuscript as accepted for publication.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding

Maybin JA, Boswell L, Young VJ, Duncan WC and Critchley HOD

The Journal of Clinical Endocrinology & Metabolism
Endocrine Society

Submitted: October 12, 2016

Accepted: December 28, 2016

First Online: January 03, 2017

Advance Articles are PDF versions of manuscripts that have been peer reviewed and accepted but not yet copyedited. The manuscripts are published online as soon as possible after acceptance and before the copyedited, typeset articles are published. They are posted "as is" (i.e., as submitted by the authors at the modification stage), and do not reflect editorial changes. No corrections/changes to the PDF manuscripts are accepted. Accordingly, there likely will be differences between the Advance Article manuscripts and the final, typeset articles. The manuscripts remain listed on the Advance Article page until the final, typeset articles are posted. At that point, the manuscripts are removed from the Advance Article page.

DISCLAIMER: These manuscripts are provided "as is" without warranty of any kind, either express or particular purpose, or non-infringement. Changes will be made to these manuscripts before publication. Review and/or use or reliance on these materials is at the discretion and risk of the reader/user. In no event shall the Endocrine Society be liable for damages of any kind arising references to, products or publications do not imply endorsement of that product or publication.

Reduced Transforming Growth Factor Beta Activity in the Endometrium of Women with Heavy Menstrual Bleeding

Maybin JA, Boswell L, Young VJ, Duncan WC and Critchley HOD

MRC Centre for Reproductive Health, University of Edinburgh, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom

Received 12 October 2016. Accepted 28 December 2016.

Endometrial TGFβ1 and Heavy Menstrual Bleeding

Context: Heavy menstrual bleeding (HMB) is common and incapacitating. Aberrant menstrual endometrial repair may result in HMB. The TGFβ superfamily contributes to tissue repair, but its role in HMB is unknown. **Objective:** We hypothesised that TGFβ1 is important for endometrial repair and women with HMB have aberrant TGFβ1 activity at menses.

Participants/Setting: Endometrial biopsies were collected from women and menstrual blood loss objectively measured (HMB >80ml/cycle, normal menstrual bleeding, NMB <80ml).

Design: Immunohistochemistry and RT-PCR examined endometrial TGFβ1 ligand, receptors and downstream SMADs in women with NMB and HMB. The function and regulation of TGFβ1 was examined using primary stromal cell culture. **Results:** *TGFB1* mRNA was maximal immediately prior to menses, but no differences were observed between women with NMB and HMB at any cycle stage. Histoscoring of *TGFB1* revealed reduced staining in the stromal compartment during menses in women with HMB ($p < 0.05$). There were no significant differences in *TGFBRI/2* or *TGFBRI/2* immunostaining. Cortisol increased activation of *TGFB1* in the supernatant of human endometrial stromal cells (HES, $P < 0.05$) via thrombospondin-1. Endometrial *SMAD2* and *SMAD3* were lower in women with HMB during menstruation ($P < 0.05$) and decreased phosphorylated *SMAD2/3* immunostaining was seen in glandular epithelial cells during the late secretory phase ($p < 0.05$). Wound scratch assays revealed increased repair in HES cells treated with TGFβ1 versus control ($P > 0.05$).

Conclusions: Women with HMB had decreased TGFβ1 and SMADs peri-menstrually. Cortisol activated latent TGFβ1 to enhance endometrial stromal cell repair. Decreased TGFβ1 activity may hinder repair of the denuded menstrual endometrium, resulting in HMB.

PRECIS: TGFβ1 and downstream SMADs were decreased in perimenstrual endometrium from women with heavy menstruation versus controls. TGFβ1 was activated by cortisol in endometrial cells and enhanced repair.

Introduction

The human endometrium is a complex and dynamic tissue. Throughout the reproductive years of a woman's life it responds to steroid hormones to prepare for implantation, shed its luminal portion in the absence of pregnancy and efficiently regenerate for the subsequent menstrual cycle. Menstruation occurs as a result of the sharp decline in progesterone as the corpus luteum regresses. This progesterone withdrawal stimulates an influx of inflammatory cells and release of matrix metalloproteinases, resulting in tissue destruction and menstrual bleeding (1,2).

The regulation of endometrial repair after shedding remains undefined. Scanning electron microscopy and hysteroscopy analysis revealed that luminal epithelial cell migration precedes stromal expansion, but that breakdown and repair occur simultaneously in adjacent sections of the human endometrium during active bleeding (3). Initiation of endometrial repair therefore occurs during the menstrual phase, when ovarian hormone levels remain low. Indeed, in the mouse model of simulated menstruation repair occurred without delay when both exogenous and endogenous estrogens were removed (4).

The transforming growth factor beta (TGF β) superfamily includes TGF β s, activins, nodal and bone morphogenic proteins (BMPs). This superfamily have been implicated in cell motility, proliferation, apoptosis, immune response and differentiation [reviewed in (5)]. Therefore, they are attractive candidates for the co-ordination of endometrial repair at menses.

TGF β is synthesised as a dimeric pre-proprotein and is released in a latent form. It is activated in a tissue specific fashion by a variety of mechanisms, including extremes of pH or via plasmin or thrombospondin-1 (6,7). Once activated, it binds to type II transmembrane serine/threonine kinase receptors, which then form a heterotetrameric complex with dimers of type I receptors. This leads to phosphorylation and activation of intracellular regulatory SMADs (SMAD-2 and -3), which in turn interact with the co-mediator SMAD-4 and translocate to the nucleus to regulate transcription of target genes. TGF β ligands and receptors are present in the human endometrium with maximal levels found during menstruation (8). TGF β ligand expression was found to be suppressed by progesterone (8), meaning endometrial induction following progesterone withdrawal is expected. Despite the low levels of circulating progesterone and estradiol at menses, local generation of steroids in the endometrium may play a vital role in menstrual physiology. Endometrial expression of the enzyme 11 β HSD1, necessary for local generation of cortisol, and the expression of the cortisol receptor GR have both been reported to be up-regulated at the time of menses (9). The role of cortisol in the regulation of TGF β remains undetermined.

Heavy menstrual bleeding of endometrial origin (HMB-E) is a common condition with a significant impact on the quality of life of otherwise healthy women (10). The financial costs to women, their families and employers are marked (11). HMB-E can be contributed, at least in part, to delayed or ineffective endometrial repair at menses. Identification of the mechanisms involved in endometrial repair and aberrations in women with HMB-E will lead to new, effective medical therapies for the many women suffering from this debilitating condition.

Herein we hypothesise that TGF β 1, its receptors and downstream SMADs are important for endometrial repair at menses and that women with HMB-E have aberrant expression of this superfamily prior to and during the menstrual phase. To investigate this we used well-categorised endometrial whole tissue biopsies from women with objectively measured normal (<80ml) and heavy (>80ml) menstrual blood loss alongside *in vitro* endometrial cell culture and functional assays.

Materials and Methods

Tissue collection

Endometrial biopsies were collected with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from 91 healthy women of reproductive age, who were predominantly White/Caucasian. Written informed consent was obtained and ethical approval granted from Lothian Research Ethics Committee (LREC/07/S1103/29). Participants were aged 22 to 50 years (median 41; mean 41). All reported regular menstrual cycles (21 to 35 days) and had not taken any exogenous hormones or used an intrauterine device for 3 months prior to tissue collection. Women with large fibroids (>3cm) and endometriosis were excluded.

Immediately after collection tissue was divided when possible and placed in (i) RNA later stabilisation solution (Ambion (Europe) Ltd., Warrington, UK) and stored at -70°C for RNA extraction, (ii) neutral buffered formalin (NBF) prior to paraffin wax embedding and (iii) phosphate buffered saline (PBS) for stromal cell extraction. If limited tissue was obtained (which often occurred with menstrual phase collection), NBF fixation was prioritised.

Menstrual stage was carefully categorised according to (i) histological appearance based on the criteria of Noyes *et al.* (12), assessed by a consultant pathologist (ii) the participant's

reported last menstrual period (LMP) and (iii) serum progesterone and estradiol levels at the time of biopsy (see supplementary methods). Consistency for all three parameters was necessary before inclusion. Six endometrial tissue samples were excluded due to inconsistent dating and one sample due to detection of hyperplasia. Biopsies were classified as proliferative (P), early-mid secretory (E-MS), late secretory (LS) or menstrual (M) for analysis (Supplementary Table 1).

Objective menstrual blood loss measurement

A subset of women (n=78) also had objective measurement of their menstrual blood loss (MBL) using the modified alkaline haematin method, as previously published (13,14). In brief, women were given the same brand of tampon and/or pad (Tampax tampons and Always towels, Proctor and Gamble, UK), with verbal and written instruction on collection. Used sanitary products were added to a measured volume of 5% sodium hydroxide. The contents were left for 24h to allow conversion of haemoglobin to haematin. During the same time period, a 1 in 200 dilution of the patient's venous blood in 5% sodium hydroxide was made and stored separately. The optical density (OD) of the samples were then measured using spectrophotometry at 546nm (A_{546}). MBL was calculated using the following equation (13):

$$\text{MBL} = \frac{(\text{OD of menstrual blood solution} \times \text{total volume of added NaOH})}{(\text{OD of venous blood} \times 200)}$$

Greater than 80ml was classified as heavy menstrual bleeding (HMB) and less than 80ml as normal (NMB).

Immunohistochemistry

5µm tissue sections were deparaffinised in xylene and rehydrated. Slides for TGFβRI and II were loaded into a Celerus Riptide de-cloaking chamber (Celerus Diagnostics Carpinteria, CA, United States) epitope retrieval was performed using Novocastra Epitope Retrieval solution Ph6 (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). Slides were loaded onto Leica Bond-Max automated immunostainer (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). Primary antibodies were applied for 2h at 37°C (see supplementary Table 2) and negative control tissues incubated with isotype-matched IgG at the same concentration as the primary antibody. The presence of antigen was visualised with Bond Polymer refine detection kit (Leica Microsystems GmbH, Ernst-Leitz-Straße, Wetzlar, Germany). TGFβ1 detection was performed on the laboratory bench after pH9 antigen retrieval. The ImmPRESS polymerised reporter system (Vector Laboratories) was utilised before liquid diaminobenzidine (DAB) kit (Zymed Laboratories, San Francisco, CA, USA) detection. Sections were counterstained with haematoxylin, dehydrated and mounted with Pertex (Cellpath plc, Hemel Hempstead, UK).

Semi-quantitative histoscore

Localisation and intensity of immunostaining was evaluated by two independent, masked observers (15). The intensity of staining was graded with a three-point scale (0 = no staining, 1 = mild staining, 2 = strong staining). This was applied to the glands and stromal cells, as well as the surface epithelium and endothelial cells where visualised (N.B. the latter two cellular components were often absent in menstrual phase tissue, accounting for the lower n-numbers in these groups). The percentage of tissue in each intensity scale was recorded (15). A value was derived for each of the cellular compartments by using the sum of these percentages after multiplication by the intensity of staining.

Cell culture

Primary human endometrial stromal (HES) cells were isolated from secretory endometrial tissue (n=6) by enzymatic digestion as previously described (16). These women met the criteria detailed above in “tissue collection” but did not undergo objective measurement of their menstrual blood loss. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FCS, 1% 200mM L-Glutamine and 500mg/ml Gentamycin.

Secretory phase HES cells from three patients with a subjective complaint of HMB and not using oral or inhaled corticosteroids were plated at 3×10^5 cells per well in 6 well plates in 10% RPMI 1640. The next day, cells were washed in PBS and incubated in serum free media overnight. Cells were then treated for 24h in duplicate with (i) vehicle (1:1000 ETOH), (ii) 1 μ M cortisol (17) or (iii) 1 μ M cortisol plus 5 μ M LSKL, a TSP-1 inhibitor (following a 2h pre-treatment with 5 μ M LSKL alone). The cell supernatant was collected for ELISA and RNA extracted from cells.

Q RT PCR

Total RNA from cells and endometrial biopsies was extracted using the RNeasy Mini Kit (Qiagen Ltd, Sussex, UK) with on column DNaseI digestion according to manufacturer's instructions. RNA samples were reverse transcribed using the Superscript VILO cDNA synthesis kit (Invitrogen, Paisley, UK) according to manufacturer's instruction with appropriate controls. Primers for each gene of interest were designed using the Universal Probe Library Assay Design Center (Roche Applied Science, Burgess Hill, UK) (see supplementary Table 3) and purchased from Eurofins (MGW Operon, Ebergsberg, Germany). PCR was carried out using ABI Prism 7900 (Applied Biosystems). Samples and controls were analysed in triplicate using Sequence Detector version 2.3 (PE Biosystems), using the comparative threshold method. Messenger RNA transcripts were normalised relative to the geomean of two appropriate housekeeping genes 18S and ATP5B as determined by geNorm assay (Primerdesign Ltd., Southampton, UK) and quantified relative to a positive human liver cDNA sample.

ELISA

A TGF- β 1 ELISA was performed using a Human TGF- β 1 Quantikine Kit (DB100B; R&D Systems) according to the manufacturer's instructions. Samples were analysed without activation and with latent TGF- β 1 activated to the immunoreactive form using 1m HCl and neutralized with 1.2m NaOH/0.5m HEPES buffer. Samples were assayed in duplicate, and after development assays were measured on a Lab Systems Multiscan EX Microplate reader at 450nm with wavelength correction at 540nm. Values were determined by standard curve analysis. Intra-assay CV was 2.5%, and the between-batch CV was 8.3% for cell culture supernatants.

Wound scratch assay

Secretory phase HES from three participants (passage <5) were seeded at 2×10^5 per well in 12 well plates in appropriate supplemented media (see above) and 16h before scratch, medium was changed to serum-free. Each well of cells was scratched with a sterile 200 μ l pipette tip, washed with PBS and then incubated in serum free media with vehicle, 1ng human recombinant TGF β 1 (Peprotech, London, UK) or 10 μ g/ml of the TGF β type I activin receptor-like kinase (ALK) receptors inhibitor SB 431542 hydrate (Sigma Aldridge, Dorset, UK) (n = 3 participants, triplicate wells for each). For each well, 4-5 images were captured along the length of each wound at 0 and 24h using an Axiovert 200M inverted microscope (Carl Zeiss, Jena, Germany). Images were analyzed using AxioVision release 4.72, and calculations of average distance closed for each sample were based on three measurements at identical positions along each wound image at 0 and 24h.

Statistical Analysis

Analysis was carried out using GraphPad Prism Software, Inc., San Diego, CA. For comparison of multiple data sets with two grouping variables (i.e. HMB vs NMB and stage of menstrual cycle, mRNA and immunohistochemistry data) a two way ANOVA was utilised, with Bonferroni's multiple comparisons test. A paired one way ANOVA with Tukey's multiple comparisons test was used to compare cell culture treatments. Tissue and cell endometrial mRNA results were expressed as the quantity relative to a comparator sample of RNA from human liver. A value of $P < 0.05$ was considered significant.

Results

There is increased transcription of *TGFB1* in the late secretory phase

TGF β 1 mRNA was examined by qRT PCR in whole endometrial biopsies from women sampled at various stages of the menstrual cycle who had objectively determined menstrual blood loss. Overall the stage of the menstrual cycle had a significant impact on *TGFB1* expression ($P = 0.0025$, $F = 5.339$), with the late secretory phase resulting in significantly higher levels of *TGFB1* than endometrium from the proliferative ($p < 0.001$) or early-mid secretory ($p < 0.01$) phases (Figure 1). There was no significant difference between the late secretory and menstrual phase. The increased transcription of TGF β 1 in the late secretory phase did not continue into the menstrual phase.

Women with HMB do not have altered endometrial *TGFB1* transcription or TGF β 1 reception

We compared the expression of *TGFB1* in women with NMB and HMB (Figure 1) and found no significant difference in *TGFB1* expression between the two groups at any cycle stage. In addition the two major TGF β 1 receptors, type I and type II, were examined in the late secretory and menstrual endometrial samples. Neither *TGFB1* nor *TGFB2* expression was significantly different in endometrium from women with HMB versus NMB (Figure 2A,B). Immunohistochemical staining revealed maximal staining of TGFBR1 in surface and glandular epithelial cells, with lower intensity staining in the stromal compartment (Figure 2C). TGFBR2 showed a similar pattern, with highest immunostaining in epithelial and endothelial cells (Figure 2D). Semi-quantitative histoscore by two masked independent observers confirmed no differences in either receptor when comparing women with HMB and NMB throughout the perimenstrual phase (Figure 2E,F).

Women with HMB have reduced perimenstrual endometrial stromal *TGFB1*

As the numerous cell types in the endometrium differentially expressed TGF β 1 receptors, we examined the localisation of TGF β 1 by immunohistochemistry. TGF β 1 could be immunolocalized to the cytoplasm of the surface epithelium, glandular epithelium, stromal cells and endothelial cells throughout the perimenstrual phase of the cycle in women with NMB (< 80 ml) and HMB (> 80 ml) (Figure 3A). Semi quantitative histoscore revealed that protein in the menstrual phase was similar to late secretory phase. There was significantly reduced TGF β 1 staining in the stromal cell compartment of endometrium from women with HMB versus those with NMB (Figure 3B). This suggests some post transcriptional regulation of TGF β 1 in stromal cells.

Cortisol increases stromal TGF β 1 activity via thrombospondin-1

To further investigate the post-transcriptional regulation of TGF β 1 we collected primary HES cells from three women in the secretory phase of the menstrual cycle for *in vitro* analysis. Peri-menstrual serum progesterone and estradiol levels were not significantly different between women with HMB or NMB (Supplementary Table 1). However, we have previously shown that cortisol is involved both in endometrial repair and the regulation of endometrial thrombospondin-1 (TSP-1) (14), a known regulator of TGF β 1 activity (6). Cortisol or cortisol plus LSKL (a TSP-1 inhibitor) produced a significant decrease in *TGFB1* expression

in HES cells ($p < 0.05$, Figure 4A) but there was no difference in the amount of latent TGF β 1 secreted, detected by pH activation of culture supernatants prior to detection of activated TGF β 1 by ELISA (Figure 4B). However analysis of unactivated cell culture supernatants revealed an increase in activation of TGF β 1 protein on treatment with cortisol, which was prevented with co-treatment of cells with the TSP-1 inhibitor LSKL ($p > 0.05$, Figure 4C). These data reveal cortisol does not increase the transcription or latent protein levels of stromal cell TGF- β 1 but has a role in the activation of latent TGF- β 1 in human endometrial stromal cells, via TSP-1.

Women with HMB have reduced perimenstrual endometrial SMAD 2/3

TGF β 1 activity increases the expression and phosphorylation of the regulatory SMADs (SMAD2 and SMAD3). These activated pSMADs then interact with the co-mediator SMAD-4 and translocate to the nucleus to regulate transcription of target genes (5). Examination of *SMAD2* and *SMAD3* expression revealed significant decreases in women with HMB versus NMB during the menstrual phase of the cycle ($P < 0.05$, Figures 5A,B). SMAD2 was significantly increased in women with HMB versus NMB during the late secretory phase. (Figure 5A). Immunohistochemical staining for phosphorylated SMAD 2/3 again revealed localisation to the glandular epithelium, surface epithelial cells, stromal compartment and endothelial cells (Figure 5C,D). Histoscore revealed a significant reduction in activated SMAD 2/3 protein levels in the endometrial glandular epithelial cells in women with HMB versus NMB during the late secretory phase of the menstrual cycle (Figure 5D).

TGF β 1 accelerates wound healing in primary endometrial cells

To examine the functional effects of increased TGF β 1 activity, primary HES were subjected to a wound scratch assay. As these cells are sources of TGF β 1 they were studied in the presence of vehicle, SB-431542 (to block endogenously stimulated phosphorylation of SMAD proteins) or TGF β 1. HES cells showed significantly increased wound closure with TGF β 1 treatment versus SB-431542 treated cells ($p < 0.05$, Figure 6A,B).

Discussion

Herein we detail significant differences in TGF β 1 downstream of local steroid action in the endometrium of women with HMB during menstruation. Endometrium from women with objectively measured HMB had decreased TGF β 1 protein levels, unaltered TGF β receptor presence and a significant reduction in both SMAD2 and -3 mRNA expression and SMAD 2/3 protein phosphorylation before/during the menstrual phase when compared to women with NMB. We provide mechanistic data supporting TGF β 1 protein activation by cortisol in endometrial cells, via thrombospondin-1. In addition, our functional studies reveal that a suboptimal TGF β response in the local endometrial environment may decrease post-menstrual repair of the stromal compartment and lead to heavy, prolonged menstrual bleeding (Figure 7).

Previous studies have detailed that TGF β 1 levels in endometrial tissue explants are suppressed by progesterone (8). These authors found secretory explants cultured for 24h in the absence of progesterone and estrogen, a milieu analogous to the menstrual phase, significantly increased *TGF β 1* mRNA. Our results support these findings, with significantly greater *TGF β 1* mRNA prior to and during menstruation when compared to the proliferative and early-mid secretory phases, consistent with up-regulation following progesterone withdrawal. We did not observe any significant difference in endometrial *TGF β 1* mRNA between women with HMB and normal blood loss during menstruation, although we acknowledge our n-numbers are small. However, we did observe significantly decreased TGF β protein in the stromal compartment of women with HMB versus NMB during

menstruation. We acknowledge that menstrual biopsy n-numbers are low but these tissues are meticulously classified and have objective measurement of participant menstrual blood loss to aid precision of data. Our results suggest differences in TGF β 1 protein in women with HMB and NMB are not due to transcriptional regulation, but that post-transcriptional regulation may be aberrant.

Interestingly, there were no significant differences in serum progesterone or estradiol levels between women with HMB and NMB. In addition, no significant differences in endometrial estradiol receptor or progesterone receptor expression were previously detected in women with measured menstrual blood loss (18). Therefore, we hypothesised that local cortisol action may influence TGF β 1 activity during menses.

TGF β is synthesised as a dimeric pre-proprotein and is released in a latent form. Thrombospondin-1 (TSP-1) is known to activate TGF β 1 and is thought to do so by inducing a conformational change in the latent protein (6). Our laboratory has previously published that women with HMB have significantly reduced endometrial TSP-1 mRNA levels when compared to women with normal bleeding (14). Previous studies from our laboratory have also found that cortisol increases TSP-1 mRNA expression in primary human endometrial stromal cells (14). Direct measurement of cortisol levels in the endometrium of women with HMB and NMB has not yet been carried out, but an enhanced local inactivation of cortisol by 11 β HSD2 may be present in the endometrium of women with heavy menses (14). 11 β HSD2 mRNA was increased 2.5 fold in women with HMB versus NMB, predicting substantially lower local cortisol concentrations. Therefore, we examined if cortisol was a local regulator of TGF β 1 activity via TSP-1. On examination of cell culture supernatants from HES cells treated with physiological levels of cortisol (17), activated TGF β 1 was significantly increased. This increase was abrogated by the addition of a TSP-1 inhibitor to culture. Interestingly, acid activation of latent TGF β 1 in the culture supernatant prior to ELISA resulted in no differences in TGF β 1 levels with any of the treatments used. This is consistent with cortisol stimulated TSP-1 production acting on latent TGF β 1 protein to increase its activity, rather than increasing the transcription or translation of TGF β 1. Indeed, cortisol and cortisol plus TSP-1 inhibitor treatment both significantly decreased *TGF β 1*. *TGF β 1* mRNA was not significantly different in the endometrium of women with NMB versus HMB during the peri-menstrual phase, but there was a trend towards increased TGF β 1 mRNA concentrations in women with HMB at this time, consistent with lower endometrial cortisol levels (14).

Next we examined the functional significance of TGF β 1 protein levels on endometrial cells. After shedding, endometrial cells migrate to cover the exposed surface of the endometrium and the stromal compartment regenerates (19). The wound scratch assay mimics this process *in vitro*, providing a means of quantifying stromal cell migration across a wounded surface. We found that TGF β 1 increased wound healing of primary stromal cell cultures. As we detected reduced phosphorylation of SMAD 2/3 in the endometrium of women with HMB versus NMB, we blocked TGF- β -mediated activation of SMAD proteins with SB 431542 and showed a decrease in stromal cell wound migration, which was significantly less than that seen with the addition of TGF β 1. We propose that women with HMB may have defective or delayed repair of the stromal cell compartment following shedding of their functional endometrium at menses.

In addition to its functional role in proliferation, it is clear that the TGF β superfamily play an important role in endothelial cell function and blood loss. Greater than 50% of TGF β 1 knockout mice die during embryogenesis due to yolk sac defects affecting vasculogenesis and resulting in vessel fragility (20). In humans, mutation of the TGF β receptor I ALKI or of the endothelial accessory receptor endoglin causes hereditary haemorrhagic telangiectasia (HHT), an autosomal dominant vascular disease (21). The resulting aberrant TGF β

superfamily signalling results in epistaxis, telangiectasia and arteriovenous malformations. Interestingly, previous histochemical and microscopic examination of endometrial blood vessels from women with normal and heavy menstrual bleeding revealed increased endothelial gaps in women with heavy loss (22). The role of the TGF β superfamily in this pathology remains to be determined, but the observational data contained herein suggest that low late secretory/menstrual TGF β 1 protein levels and decreased pSMAD 2/3 may be involved. Previous results from our centre support a role for TGF β 1 in the generation of vasoactive factors in women with endometriosis (23,24) and it may have a similar, if more regulated, role in the endometrium to ensure physiological menstruation.

We have previously shown that cortisol is angiostatic, preventing endothelial tube-like structure formation *in vitro* (14). Furthermore, SiRNA silencing of TSP-1 in uterine endothelial cells reversed the anti-angiogenic effect. In combination with data contained herein, we propose that cortisol may activate endometrial TGF β 1 via TSP-1 during menses to prevent an excessive angiogenic response and increase vascular integrity. Further experiments are required to definitively test this hypothesis.

In conclusion, we show that women with objectively measured HMB have decreased endometrial TGF β 1 protein and downstream SMADs during the late secretory/menstrual phase when compared to women with NMB. This may partially explain the increased menstrual blood loss experienced by many women. In addition, we show that cortisol has a mechanistic role in the activation of endometrial TGF β 1 at this time (Figure 7). Our *in vitro* results are consistent with TGF β 1 having a functional role in repair of the denuded endometrial surface at menstruation and we propose that women with HMB may benefit from therapies that increase TGF β during menses.

Acknowledgements

We thank Ronnie Grant for assistance with figure preparation, Sheila Milne for manuscript formatting, Catherine Murray and Sharon McPherson for help with patient recruitment and Reena Murgai, Alison Murray and Moira Nicol for excellent technical support. University of Edinburgh undergraduate student Irene Sucquart performed some of these studies.

Corresponding author: Dr Jacqueline A Maybin, MRC Centre for Reproductive Health, University of Edinburgh, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom, E mail:

Jackie.maybin@ed.ac.uk

Grants: This study was funded by The Society of Endocrinology and the Barbour Watson Fund with additional support from the Wellcome Trust (100646/Z/12/Z) and Medical Research Council (G1002033, MR/N022556/1).

Disclosure Statement: JAM, LB, VJY have nothing to disclose. HODC has clinical research support for laboratory consumables and staff from Bayer Pharma Ag and provides consultancy advice (but with no personal remuneration) for Bayer Pharma Ag, PregLem SA, Gideon Richter, Vifor Pharma UK Ltd, AbbVie Inc. WCD has received research funding from GSK for an unrelated project.

References

1. Marbaix E, Kokorine I, Moulin P, Donnez J, Eeckhout Y, Courtoy PJ. Menstrual breakdown of human endometrium can be mimicked *in vitro* and is selectively and reversibly blocked by inhibitors of matrix metalloproteinases. *Proc Natl Acad Sci U S A* 1996; 93:9120-9125.
2. Slayden OD, Brenner RM. A critical period of progesterone withdrawal precedes menstruation in macaques. *Reprod Biol Endocrinol* 2006; 4 Suppl 1:S6.

3. Garry R, Hart R, Karthigasu KA, Burke C. A re-appraisal of the morphological changes within the endometrium during menstruation: a hysteroscopic, histological and scanning electron microscopic study. *Hum Reprod* 2009; 24:1393-1401.
4. Kaitu'u-Lino TJ, Morison NB, Salamonsen LA. Estrogen is not essential for full endometrial restoration after breakdown: lessons from a mouse model. *Endocrinology* 2007; 148:5105-5111.
5. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007; 8:970-982.
6. Schultz-Cherry S, Murphy-Ullrich JE. Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *J Cell Biol* 1993; 122:923-932.
7. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu Rev Cell Biol* 1993; 9:541-573.
8. Gaide Chevronnay HP, Cornet PB, Delvaux D, Lemoine P, Courtoy PJ, Henriot P, Marbaix E. Opposite Regulation of Transforming Growth Factors-Beta 2 and -Beta 3 Expression in the Human Endometrium. *Endocrinology* 2007.
9. McDonald SE, Henderson TA, Gomez-Sanchez CE, Critchley HO, Mason JI. 11Beta-hydroxysteroid dehydrogenases in human endometrium. *Molecular and cellular endocrinology* 2006; 248:72-78.
10. Munro MG, Critchley HO, Broder MS, Fraser IS. FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nonpregnant women of reproductive age. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 2011; 113:3-13.
11. Cote I, Jacobs P, Cumming D. Work loss associated with increased menstrual loss in the United States. *Obstet Gynecol* 2002; 100:683-687.
12. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Fertil Steril* 1950; 1:3-25.
13. Warner PE, Critchley HO, Lumsden MA, Campbell-Brown M, Douglas A, Murray GD. Menorrhagia I: measured blood loss, clinical features, and outcome in women with heavy periods: a survey with follow-up data. *Am J Obstet Gynecol* 2004; 190:1216-1223.
14. Rae M, Mohamad A, Price D, Hadoke PW, Walker BR, Mason JI, Hillier SG, Critchley HO. Cortisol inactivation by 11beta-hydroxysteroid dehydrogenase-2 may enhance endometrial angiogenesis via reduced thrombospondin-1 in heavy menstruation. *J Clin Endocrinol Metab* 2009; 94:1443-1450.
15. Aasmundstad TA, Haugen OA, Johannesen E, Hoe AL, Kvinnsland S. Oestrogen receptor analysis: correlation between enzyme immunoassay and immunohistochemical methods. *J Clin Pathol* 1992; 45:125-129.
16. Kane N, Jones M, Brosens JJ, Saunders PT, Kelly RW, Critchley HO. Transforming Growth Factor- β 1 Attenuates Expression of Both the Progesterone Receptor and Dickkopf in Differentiated Human Endometrial Stromal Cells. *Mol Endocrinol* 2008; 22:716-728.
17. Harlow CR, Jenkins JM, Winston RM. Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. *Fertil Steril* 1997; 68:48-53.
18. Critchley HO, Abberton KM, Taylor NH, Healy DL, Rogers PA. Endometrial sex steroid receptor expression in women with menorrhagia. *Br J Obstet Gynaecol* 1994; 101:428-434.
19. Ludwig H, Spornitz UM. Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodeling. *Ann N Y Acad Sci* 1991; 622:28-46.

20. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 1995; 121:1845-1854.
21. Abdalla SA, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *J Med Genet* 2006; 43:97-110.
22. Mints M, Hultenby K, Zetterberg E, Blomgren B, Falconer C, Rogers R, Palmblad J. Wall discontinuities and increased expression of vascular endothelial growth factor-A and vascular endothelial growth factor receptors 1 and 2 in endometrial blood vessels of women with menorrhagia. *Fertil Steril* 2007; 88:691-697.
23. Young VJ, Brown JK, Maybin J, Saunders PT, Duncan WC, Horne AW. Transforming growth factor-beta induced Warburg-like metabolic reprogramming may underpin the development of peritoneal endometriosis. *J Clin Endocrinol Metab* 2014; 99:3450-3459.
24. Young VJ, Brown JK, Saunders PT, Duncan WC, Horne AW. The peritoneum is both a source and target of TGF-beta in women with endometriosis. *PLoS One* 2014; 9:e106773.

Figure 1. *TGFβ1* in the human endometrium. TGFβ1 mRNA expression in endometrium from across the menstrual cycle in women with HMB (blood loss >80ml) and NMB (blood loss <80ml). P proliferative, E/MS early-mid secretory, LS late secretory, M menstrual. ***P<0.001, **P<0.01.

Figure 2. TGFβRI and TGFβRII in the human endometrium before and during menstruation. (A) TGFβRI mRNA expression in endometrium from women with normal (NMB; <80ml) and heavy (HMB; >80ml) menstrual bleeding during the late secretory (LS) and menstrual (M) phases (B) TGFβRII mRNA expression (C) Immunohistochemical staining of TGFβRI in endometrium from the late secretory phase. SE: surface epithelium, GE: glandular epithelium, St: stromal cell compartment, arrow: endothelial cells (D) Immunohistochemical staining of TGFβRII in endometrium from the late secretory phase, inset: negative control (E) Immunohistochemical histoscore of TGFβRI in human endometrium from women with heavy and normal bleeding during the late secretory and menstrual phases (F) Immunohistochemical histoscore of TGFβRII in human endometrium from women with heavy and normal bleeding during the late secretory and menstrual phases. (N.B. lower n-numbers appear in SE and endothelial cell scoring due to the inability to identify these cells in some tissues).

Figure 3. Immunohistochemistry for TGFβ1 in human endometrium from the perimenstrual phase. (A) Staining of late secretory and menstrual phase endometrium from women with HMB (>80ml) and NMB (<80ml). GE glandular epithelium, SE surface epithelium, St stromal compartment, arrows indicate endothelial cells. Inset: negative control (B) Semi quantitative histoscore of TGFβ1 immunohistochemistry staining.

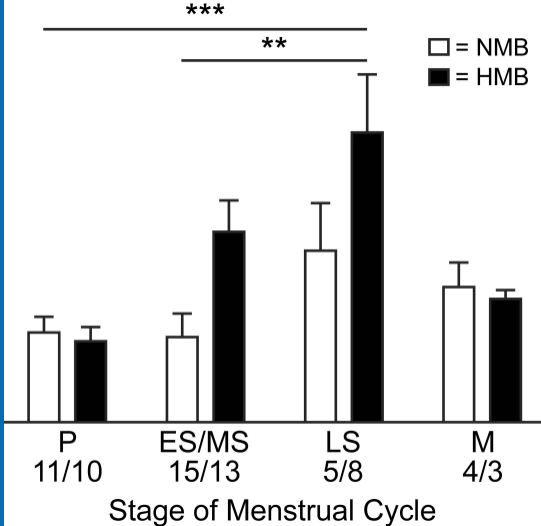
Figure 4. The regulation of TGFβ1 by cortisol in primary human endometrial stromal cells. (A) TGFβ1 mRNA after 24h treatment with vehicle, cortisol (1μM) or cortisol (1μM) plus a thrombospondin-1 inhibitor (LSKL 5μM) (B) Active TGFβ1 protein levels in experimental culture supernatants following pre-ELISA acid activation of latent TGFβ1 (C). Active TGFβ1 protein levels in the same culture supernatants without pre-ELISA acid activation. (*P<0.05).

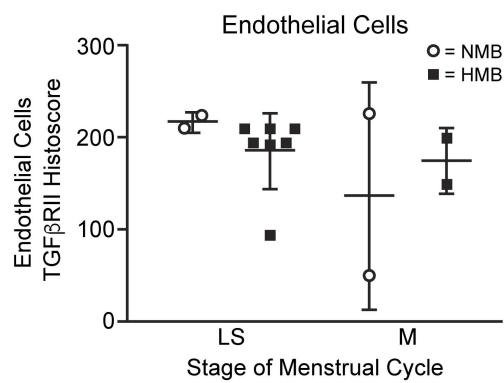
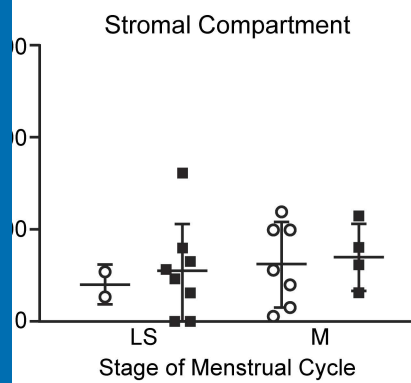
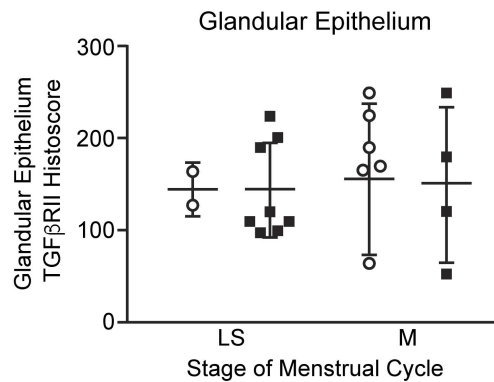
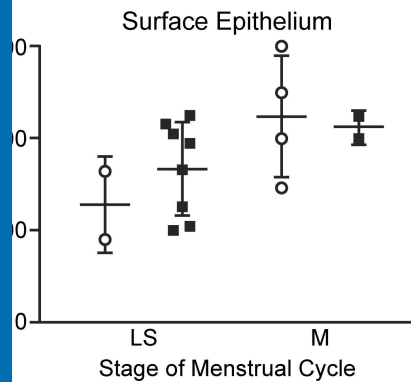
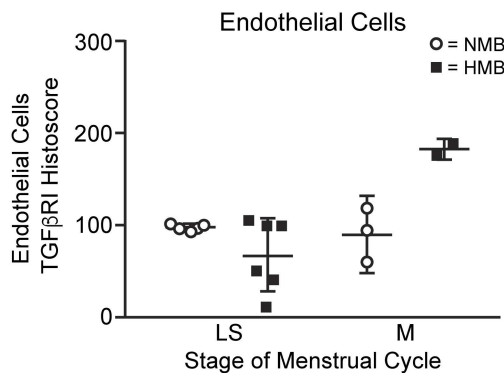
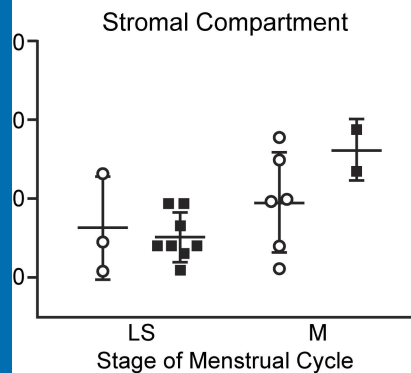
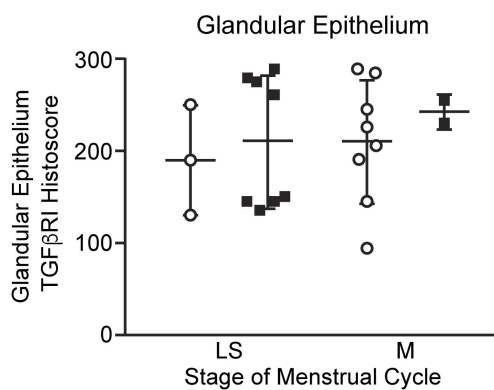
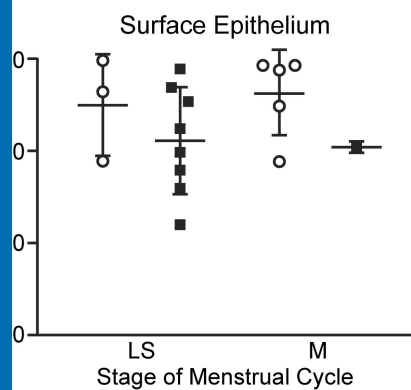
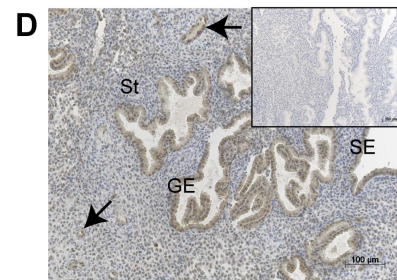
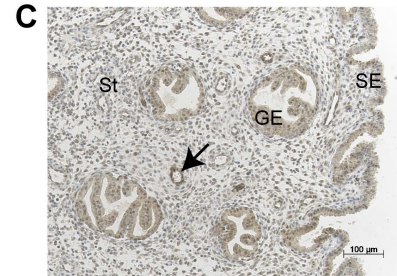
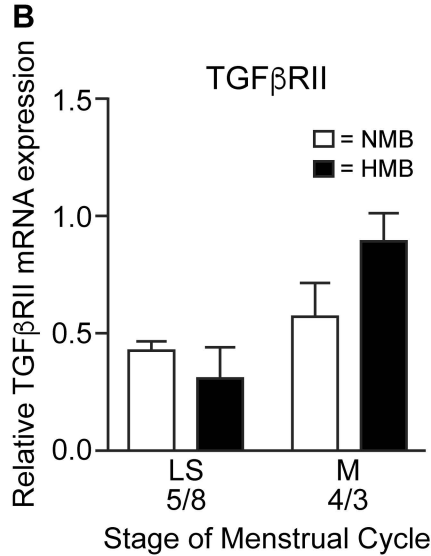
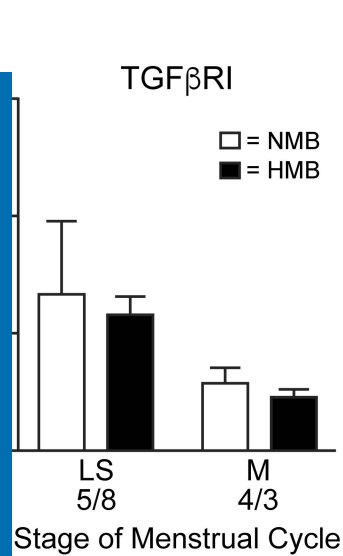
Figure 5. SMAD2/3 in the human endometrium before and during menstruation. (A) SMAD2 mRNA expression in endometrium from women with normal (NMB; <80ml) and heavy (HMB; >80ml) menstrual bleeding during the late secretory (LS) and menstrual (M)

phases (B) SMAD3 mRNA expression in endometrium from women with NMB and HMB in the LS and M phases (C) Phosphorylated SMAD2/3 immunohistochemical staining in late secretory endometrium from a woman with NMB. Inset: negative control. SE: surface epithelium, GE: glandular epithelium, St: stromal cell compartment, arrow: endothelial cells (D) Phosphorylated SMAD2/3 immunohistochemical staining in late secretory endometrium from a woman with HMB (E) Histochemical staining for phosphorylated SMAD2/3. *P<0.05.

Figure 6. The effect of TGFβ1 on human endometrial cell wound repair. (A) Average wound scratch closure distance (scratch distance at 0h minus scratch distance at 24h) in human primary stromal endometrial cells after treatment with vehicle, the Alk receptor inhibitor SB431542 or 1ng TGFβ1. (B) Images of wound scratch in HES cells treated with 10μg/ml SB431542 for (i) 0h (ii) 24h and treated with 1ng TGFβ1 (iii) 0h and (iv) 24h.

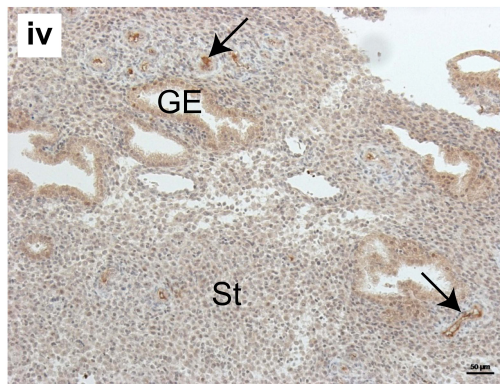
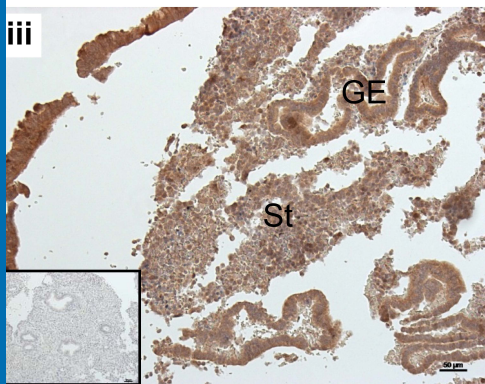
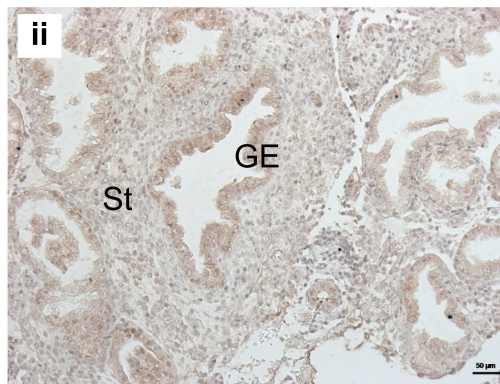
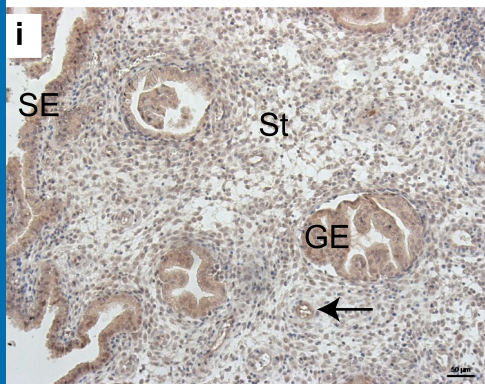
Figure 7. Proposed role of TGFβ1 in the human endometrium at menstruation. Red stars represent findings in women with HMB and potential impact on endometrial function.



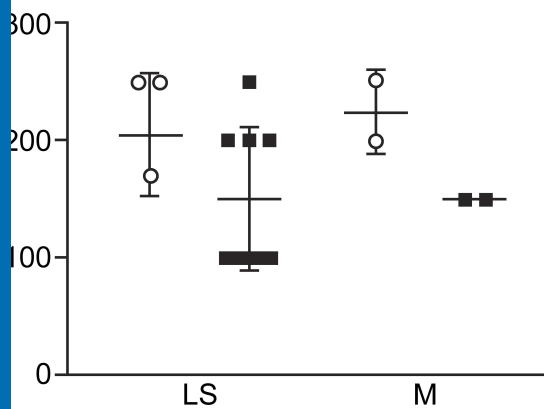


NMB (<80ml)

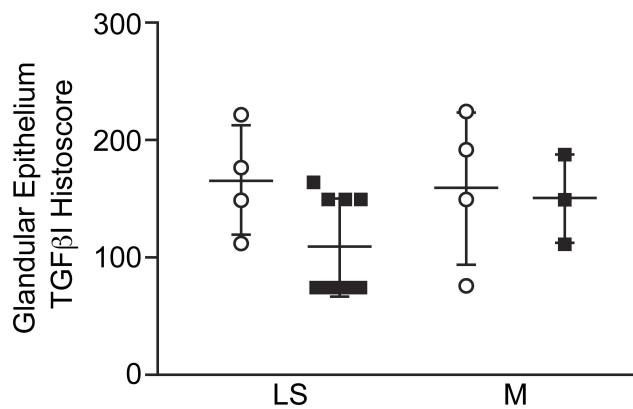
HMB (>80ml)



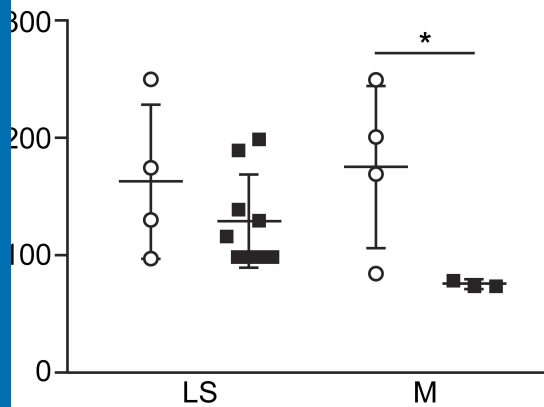
Surface Epithelium



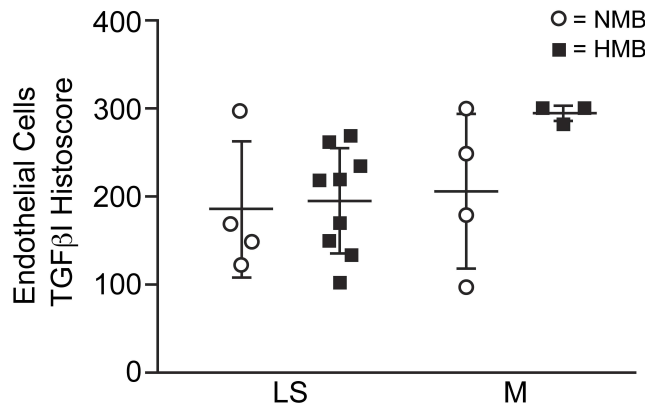
Glandular Epithelium

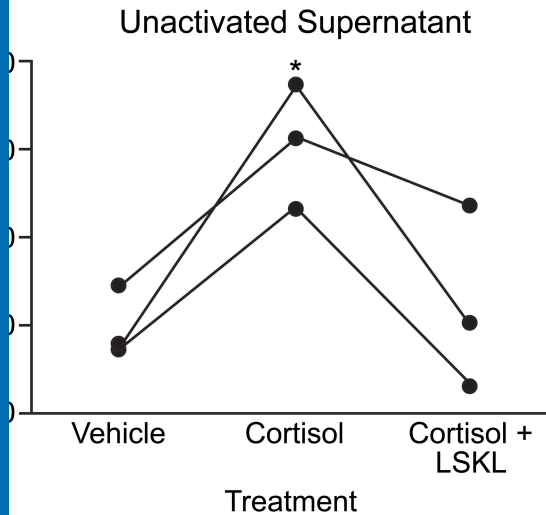
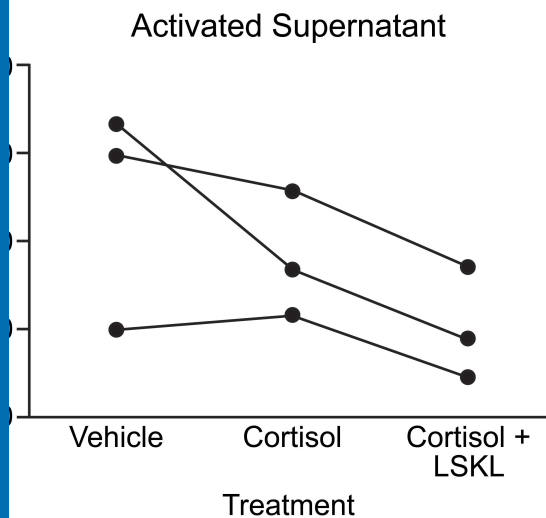
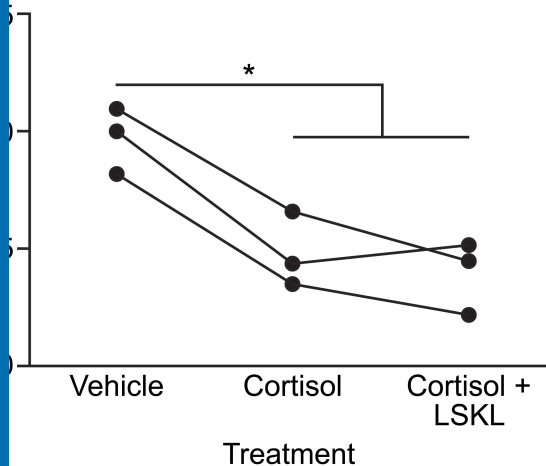


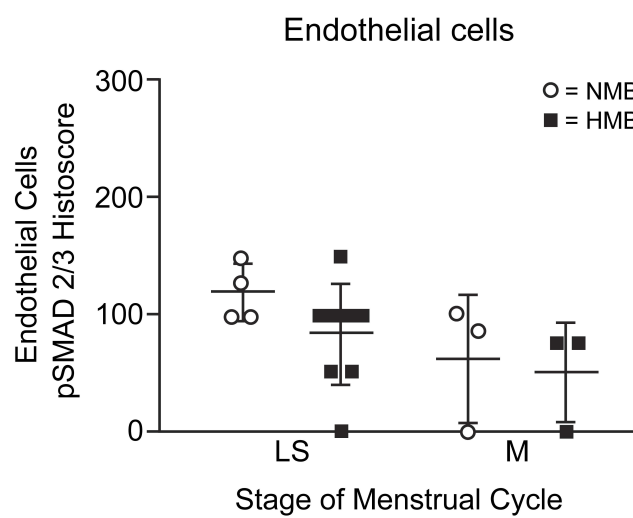
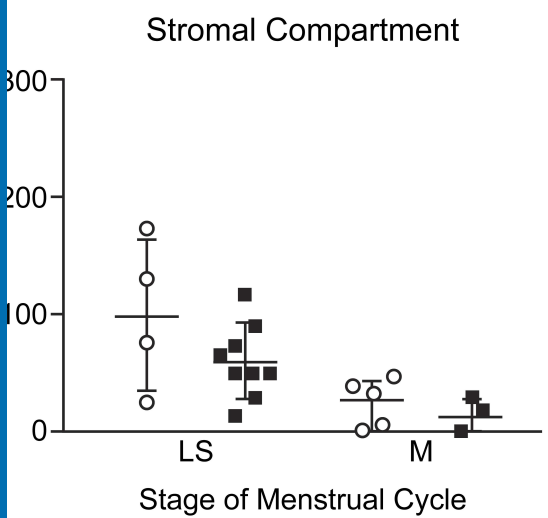
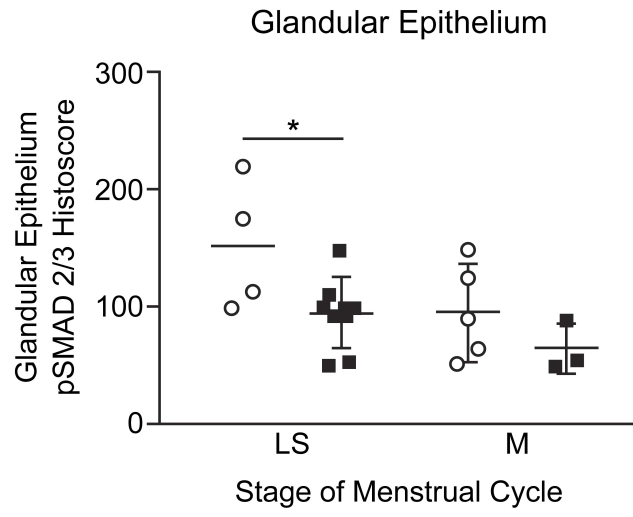
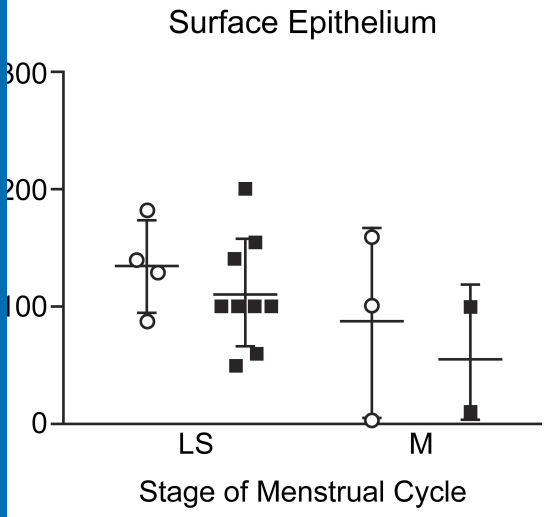
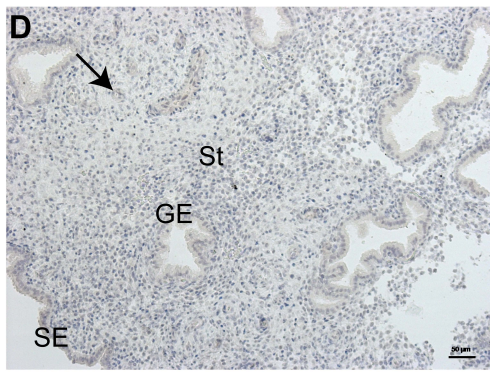
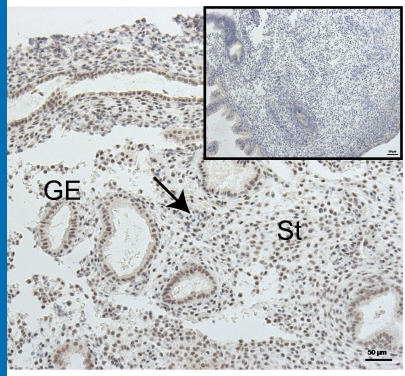
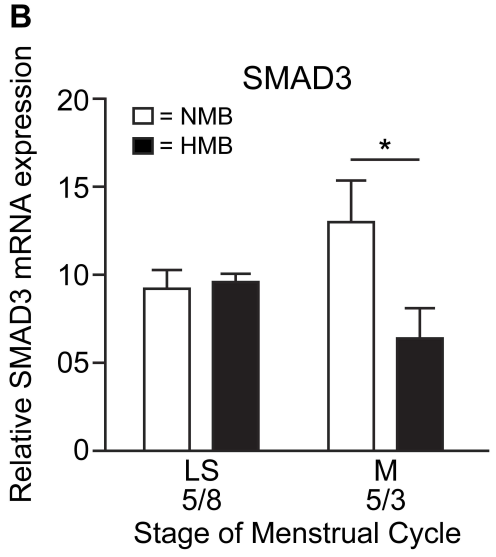
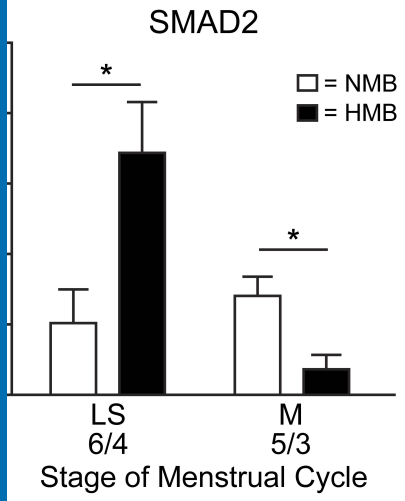
Stromal Compartment

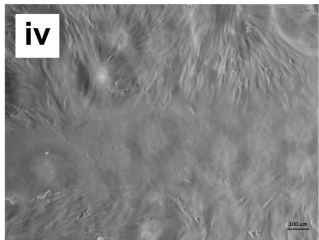
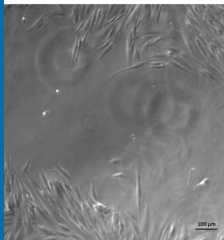
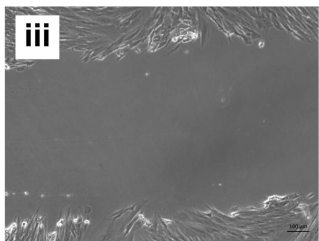
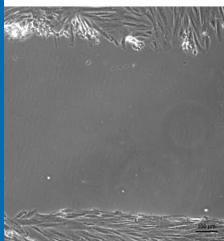
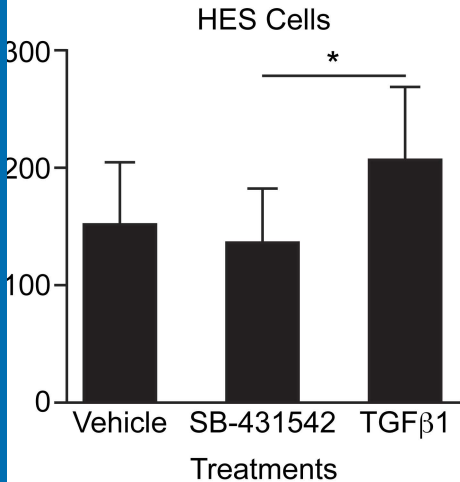


Endothelial Cells





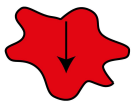




Local Cortisol



TSP-1



TGFβ1

Activated TGFβ1

KEY

- P = phosphorylation
- HES = human endometrial stromal cells
- GE = glandular epithelial cells
- TSP-1 = thrombospondin-1
- TGFβ1 = transforming growth factor beta 1
- TGFβR = transforming growth factor beta receptor

