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Epithelial and stromal-specific immune pathway activation in the murine endometrium *post-coitum*

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Abstract:

The endometrium is a dynamic tissue, demonstrating cyclical growth/remodelling in preparation for implantation. In mice, seminal constituents trigger mechanisms to prepare the endometrium, a process dubbed 'seminal priming' which modifies immune system components and mediates endometrial remodelling in preparation for pregnancy. An array of cytokines has been reported to mediate this interaction, although much of the literature relates to *in vitro* studies on isolated endometrial epithelial cells. This study measured changes in immune-related gene expression in endometrial epithelial and stromal cells *in vivo* following natural mating. CD1 mice were naturally mated and sacrificed over the first four days *post-coitum* (*n*=3 each day). Endometrial epithelial and stromal compartments were isolated by laser capture microdissection. Labelled cRNA was generated and hybridised to genome-wide expression microarrays. Pathway analysis identified several immune-related pathways active within epithelial and stromal compartments, in particular relating to cytokine networks, matrix metalloproteinases and prostaglandin synthesis. Cluster analysis demonstrated that expression of factors involved in immunomodulation/endometrial remodelling differed between the epithelial and stromal compartments in a temporal fashion. This study is the first to examine the disparate responses of the endometrial epithelial and stromal compartments to seminal plasma *in vivo* in mice, and demonstrates the complexity of the interactions between these two compartments needed to create a permissive environment for implantation.

Introduction

The endometrium is a dynamic tissue which undergoes cyclical growth and remodelling to a greater or lesser degree in different species in preparation for blastocyst implantation. In the mouse, seminal constituents are thought to trigger the mechanisms preparing the endometrium for implantation in a process dubbed 'seminal priming' (Figure I), which can modify immune system components and influence subsequent events in pregnancy (Robertson, et al. 2013). The endometrium responds rapidly to seminal fluid exposure by triggering an inflammatory cascade which is soon followed by an influx of leukocytes (Sharkey, et al. 2012). Seminal plasma proteins interact with oestrogen (E_2)-primed uterine epithelial cells, resulting in the synthesis of a large array of cytokines and other immunoregulatory factors, such as granulocyte macrophage colony stimulating factor (CSF2), interleukin (IL)6, IL8, monocyte chemoattractant protein (CCL2), matrix metalloproteinases (MMPs) and prostaglandins (PGs) (Robertson, et al. 1996a, Sanford, et al. 1992). Within hours of this rise in cytokine production, inflammatory cells invade the cervix as the primary site of semen deposition in humans, pigs, rabbits and mice, although in rodents this inflammatory response can be seen throughout the endometrium (Claus 1990, Lovell and Getty 1968, McMaster, et al. 1992, Pandya and Cohen 1985, Robertson 2005, Rozeboom, et al. 1998, Taylor 1982). Myeloid lineage cells such as monocytes/macrophages, dendritic cells and granulocytes of circulatory origin extravasate to accumulate in uterine stromal tissue, with macrophages, neutrophils and other granulocytes in particular further traversing the epithelium to accumulate in the uterine cavity (Bischof, et al. 1994, De, et al. 1991, McMaster, et al. 1992). This initial neutrophil response resolves prior to implantation, with a purported concurrent drop in cytokine profiles and rise in progesterone (P_4) (Robertson, et al. 1996a). Endometrial leukocytes, however, persist for several days (O'Leary, et al. 2004).

Cytokines such as CSF2 (Robertson, et al. 1996b, Robertson and Seamark 1990), leukaemia inhibitory factor (LIF), CCL2 (Boomsma, et al. 2009, Meter, et al. 2005, Wood, et

al. 1997, Wood, et al. 1999), regulated upon activation normal T cell expressed and secreted (CCL5) (Altman, et al. 1999, Arima, et al. 2000, Wood, et al. 1997) and TGFB have been shown to be expressed in endometrial cell compartments *post-coitum*, although much of these data relate to *in vitro* models. However, most of the peri-implantation tissue remodelling (both prior to and during decidualisation) appears to be mediated by MMPs (Aplin 1997, Tang, et al. 2005). Several MMPs have been implicated, with macrophages being the main source of these proteases, which they produce in response to the prevailing cytokine and prostaglandin milieu (Goetzl, et al. 1996). However, MMP function is reasonably species-specific: in women, these enzymes are the main mediators of endometrial shedding whereas this process is largely absent in rodents, where endometrial remodelling merely involves functional regression (Marbaix, et al. 1996, Tang, et al. 2005).

Alongside endometrial remodelling, a concurrent modification of the maternal immune system occurs in order to promote maternal immunotolerance of the paternal antigens displayed by the implanting blastocyst. Following initial semen deposition in the female reproductive tract, paternal antigens are phagocytosed, processed and presented to maternal $CD4^+$ and $CD8^+$ T cells by antigen presenting cells in the para-aortic lymph nodes (Johansson, et al. 2004, Moldenhauer, et al. 2009). This induces a functional anergy/hyporesponsiveness in these cells which promotes conceptus allograft tolerance (Tafuri, et al. 1995), in line with a concurrent expansion of the $CD4^+CD25^+Foxp3^+$ regulatory T cell (Treg) pool (Aluvihare, et al. 2004, Shima, et al. 2010). The strength of this maternal response depends on seminal plasma composition, particularly paternal antigen content and TGFB concentrations (Robertson, et al. 2009). Seminal plasma TGFB in humans and swine is largely accounted for by the TGFB1 isoform (although TGFB2 and 3 are also present) which is secreted in an inactive form requiring activation in the acidic environment of the vagina (Politch, et al. 2007). The activation of TGFB1 induces naïve $CD4^+CD25^-$ T cells to differentiate into $Foxp3^+$ suppressor/anergic T cells (Chen, et al. 2003). The prostaglandin component of seminal plasma may also synergise with TGFB in this regard since PGE_2

enhances this inhibitory effect and has been shown to induce a regulatory phenotype in human $CD4^+CD25^-$ T cells *in vitro* (Baratelli, et al. 2005). PG synthesis is also induced by seminal plasma in the porcine uterus, with concurrent angiogenesis extending throughout the preimplantation period (Kaczmarek, et al. 2010, Kaczmarek, et al. 2013).

Although individual immune mediators of the endometrial response to seminal plasma have been identified, few studies have focussed on trying to paint a comprehensive picture of the large numbers of mediators involved, particularly in terms of characterising the global immunomodulatory responses involved at the level of the transcriptome. Various studies have focussed on profiling gene expression over the course of the implantation window (particularly in women following ovarian stimulation) in order to identify those genes most likely to be involved in the establishment of endometrial receptivity prior to embryo transfer and in relation to the implantation problems allied to endometriosis (Burney, et al. 2007, Giudice 2004, Kao, et al. 2003, Kao, et al. 2002, Riesewijk, et al. 2003). To date, however, only one has explored global gene expression in the cervix following stimulation with seminal plasma: Sharkey and co-workers (2012) performed expression microarray analysis on biopsies of pre- and post-coital human cervix and reported that seminal fluid activated pathways including the inflammatory response, immune response, immune cell trafficking, cell movement, and antigen presentation. Within these various pathways, CSF2, IL6 and IL8 mRNA were prominently upregulated *post-coitum*, as were COX-2 and various MMPs, suggesting active leukocyte recruitment and ECM remodelling (Sharkey, et al. 2012). Unfortunately, these observations were based only on a single time point in this putatively dynamic process. The aim of the present study was therefore to characterise the endometrial inflammatory response to seminal plasma *in vivo* in detail throughout the pre-implantation period by using a genome-wide screening approach using a murine model. Furthermore, the individual response of the epithelial and stromal cellular compartments to seminal plasma was examined to identify differences in gene expression.

Materials and Methods

Mouse husbandry and mating protocol

Ethical approval was not required for this study as all work was conducted in compliance with the ethical and legislative framework set out in the Animals (Scientific Procedures) Act, 1986 (UK). Individually housed male (aged 10-12 weeks), and group housed (10 per cage) nulliparous female (aged 6 weeks) CD1 mice were used for these experiments. Animals had *ad libitum* access to water and Standard Expanded Beekay diet (B&K, Grimston, Aldborough, UK). The lighting cycle was 14h:10h light:dark, (05:30 on; 19:30 off) and humidity and temperature were maintained at 55-65% and 21.5±1°C, respectively. Whitten effect synchronised females had their oestrus confirmed by vaginal cytology prior to being naturally mated to CD1 stud males (1 female:1 male). Five successful copulations were recorded prior to leaving pairs together overnight in order to confirm successful intercourse. Females were then group housed according to time of mating, before being sacrificed under Schedule 1 of the Animals (Scientific Procedures) Act, 1986 at 24, 48, 72 and 96 hours post first coitus ±1 hour (*n*=3 per group). Controls were provided by naturally cycling oestrus and dioestrus females (again assessed on the basis of vaginal cytology, *n*=3 per group).

Uterine tissue processing and staining

Within five minutes of culling, the ovaries, uterine horns and vagina were mounted in optimal cutting compound (OCT), frozen in isopentane slush in a liquid nitrogen bath before storage at -80^{o}C. Specimens were later sectioned (12µm) at -16^{o}C on a Leica CM3050S Cryostat (Leica Biosystems, UK). Sections were mounted onto Arcturus polyethylene naphthalate (PEN) membrane glass slides (Applied Biosystems, CA, USA) and stained with buffered ethanolic cresyl violet staining solution, as previously described, with minor modifications (Cummings, et al. 2011). Briefly, sections were rehydrated in a series of ethanol washes (95, 75 and 50%) prior to staining with cresyl violet in 75% ethanol buffered with 20 mM Tris-HCl pH 8.0 for 40 seconds. Sections were then dehydrated in a series of ethanol washes (50, 75, 95 and 100%). All solutions were prepared using RNAse free water and molecular grade

absolute ethanol. Slides were stored at -80^{o}C under dessicant prior to laser capture microdissection (LCM).

Laser capture microdissection

LCM of selected groups of cells was performed on a Zeiss Palm® Microbeam UV laser capture microscope, equipped with PALM@Robo software version 3.2 (Carl Zeiss, Herts, UK). Samples were captured with cut energy set to 51-60% and laser speed of 20µm/s. Excised areas were captured into adhesive cap tubes (Carl Zeiss, Herts, UK), with the average area excised per sample being $3.23x10^{6}$ μm^{2}. Both lumenal epithelial and stromal compartments were independently microdissected from the same tissue specimens in order to avoid the risk of RNA cross-contamination (Supplementary Figure I).

RNA preparation and microarray analysis

RNA extraction was performed using Qiagen RNeasy plus micro extraction kits (Qiagen, UK). The extraction protocol was based on the manufacturer's instructions with minor modifications; one extra RPE and 80% ethanol wash step was incorporated in order to reduce impurity carryover and improve RNA quality. Samples were eluted from the spin column by addition of 14µl RNAse-free water, with 0.5µl RNASecure (Ambion) immediately added to the eluate, which was then heated to 60°C for 10 minutes to inactivate RNase. Samples were concentrated using a Savant SpeedVac® concentrator (Eppendorf, Hamburg, Germany) for 30 minutes or until dry, then resuspended in 4µl RNAse-free water and stored at -80^{o}C until use. RNA quality and quantity was assessed using Agilent RNA 6000 Nano kit (Agilent, UK) on a 2100 Bioanalyser (Agilent, UK) equipped with 2100 Expert software using the RNA Eukaryote Total RNA Nano function, only samples with a RIN value of 7 and above were considered suitable for fluorescent cRNA generation.

Fluorescent cRNA was prepared using Agilent One Colour Low Input Quick AMP labelling kit with 10ng input RNA. Purification of RNA was performed using the RNeasy Mini kit (Qiagen

UK) following the manufacturer's instructions. Replicates were hybridised in different batches to avoid experimental bias. Fluorescent cRNA quality was assessed using a NanoDrop 1000 (Thermo-Scientific UK). Cyanine (Cy3) concentration (pmol/µl), RNA 260/280 ratio and cRNA concentration (ng/µl) were also recorded, and yield and specific activity were calculated for each sample. Samples were hybridised to Agilent SurePrint G3 Mouse GE 8x60k microarrays as per manufacturer's instructions. Hybridisation was performed in a hybridisation oven (Agilent, UK) for 17 hours at 65^{o}C and a rotation speed of 20rpm. Subsequent washes were completed within 1 hour. Slides were scanned immediately to reduce potential variations in signal intensities due to environmental contaminants.

Scanning and feature extraction

Microarrays were scanned using an Agilent 'C' scanner (Agilent, UK), with a scan resolution of 3µm. Feature extraction was performed using Feature Extraction version 11.0.1.1 (Agilent Technologies, UK). Sample quality was assessed utilising inbuilt quality control metrics. Eleven of these were applied to the microarray, covering aspects such as alignment, signal intensity and reproducibility, providing a range within which a microarray is considered 'good'. Microarrays falling outside of the manufacturer's 'good' criteria were evaluated for exclusion from the final analysis.

Data analysis

Data were visualised and analysed using GeneSpring version 12.5 (Agilent Technologies, UK). Data were normalised to the 75th percentile signal intensity of all probes on the array as recommended by the manufacturer. Samples were examined by the inbuilt principal components analysis (PCA) to identify major outliers. Sample size and desired fold difference were calculated using the Microarray Sample Size Computation tool available at http://bioinformatics.mdanderson.org/microarraysamplesize/. The calculation was based on 55821 entities, with an acceptable false positive rate of 5%, power (percentage of differentially expressed entities likely to be detected by the experiment) of 0.8, and a

standard deviation of 0.7. Due to the sample size, a 5-fold change cut-off was applied to the data. Pathway analysis was performed using the condition averages in order to identify immune pathways active in the murine endometrium. *P* values were corrected for multiple comparisons using the Benjamini and Hochberg false discovery rate (FDR). The results from the pathway analysis were used in subsequent cluster analyses in IBM SPSS (Version 19). The analysis was performed for epithelium and stroma individually, based on a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Identified mRNA species in each cluster were visualised in GraphPad Prism (Version 6).

Results

Pathway analysis

Pathway analysis revealed 217 curated pathways active within the murine uterine tract. Of these pathways, 46 were significant at $P<0.05$, with significance defined by the inbuilt GeneSpring algorithm which created a composite score of the number of mRNA species matched and the extent of the response of those species (Table 1). Those relating to immune networks were selected in both endometrial epithelial and stromal cell compartments active within the murine endometrium: *Prostaglandin synthesis and regulation* ($P<0.001$), *Cytokines and inflammatory response* ($P<0.001$), *MMPs* ($P=0.01$), *Inflammatory response pathway* ($P<0.05$), and the *TGFB signalling pathway* ($P<0.05$).

Cluster analysis – epithelial compartment

Genes encoding cytokine proteins and MMPs highlighted in the pathway analysis were selected for subsequent cluster analysis. These elements fell into 8 clusters (Figure II). Cluster 1 was defined by peaks in expression on days 1 and 3 post mating with reduced expression on day 2 (*Cxcl3, Il10, Il12a, Mmp9, Mmp20, Mmp25, Tnf).* Cluster 2 exhibited a similar pattern of peaks on days 1 and 3, with a less dramatic reduction in expression on day 2 (*Bmp4, Egf, Il7, Il13, Mmp1a, Mmp3, Mmp16, Mmp28).* Cluster 3 generally showed a peak in expression at oestrus and day 2 post mating with a reduction in expression on day 1 (*Ccl5, Csf3, Il1b, Il15, Inhb1, Mmp11, Mmp14, Mmp23, Thbs*). Cluster 4 demonstrated a slight peak at dioestrus and day 1 post mating (*Ifng, Mcp1, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3).* Cluster 5 showed a dip in expression at oestrus, while remaining relatively constant at other time points (*Csf2, Cxcl2, Il2, Il4, Il5, Il12b, Itbp1, Mmp10, Mmp12, Mmp13, Mmp21, Mmp24).* Cluster 6 exhibited minor peaks on days 2 and 4 post mating, although many elements showed large variation (*Cxcl1, Il1a, Lif, Mmp8, Mmp27).* Cluster 7 was characterised by peaks at dioestrus, day 1 and day 4 post mating, with a dramatic reduction in expression at day 2 and 3 (*Csf1t1, Csf1t3, Mmp7).* Finally, cluster 8 elements exhibited

huge variations in expression at each time point, and therefore could not be ascribed to any particular pattern.

Cluster analysis – stromal compartment

mRNA entities fell into 7 clusters (Figure III). Cluster 1 comprised three mRNA species (*Csf1t1, Csf3, Mmp23)* which exhibited a peak at oestrus. Cluster 2 was characterised by a slight increase in expression on day 1 post mating, with a subsequent decrease on days 2, 3 and 4 (*Bmp4, Csf1t3, Csf2, Cxcl2, Cxcl3, Egf, Ifnb1, Il1a, Il2, Il4, Il5, Il6, Il7, Il10, Il11, Il12a, Il12b, Il13, Mmp1a, Mmp3, Mmp8, Mmp10, Mmp13, Mmp12, Mmp16, Mmp20, Mmp21, Mmp24, Mmp25, Mmp28, Tnf*). Cluster 3 comprised only Mmp7, which showed highest expression at oestrus with lowest expression on day 3 post mating. Cluster 4 increased in expression post mating and remained high on days 2, 3 and 4 (*Ifng, Il15, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3*). Cluster 5 peaked on days 1 and 2 post mating with a subsequent decrease on days 3 and 4 (*Ccl5, Inhb1, Il1b, Mcp1, Mmp9, Thbs1*). Cluster 6 peaked on day 1 with a subsequent fall to baseline levels of expression (*Mmp11, Mmp14*). Cluster 7 was characterised by peaks in expression at dioestrus and day 3 post mating (*Cxcl1, Itbp1, Lif, Mmp27*).

Differences in endometrial epithelial and stromal expression

Many mRNA species displayed differences in expression between endometrial epithelial and stromal cells despite exhibiting similar pathway involvement. The majority showed greater relative expression profiles in stromal cells as compared to epithelial cells, with the exception of *Mmp7* and elements of the prostaglandin pathway which were consistently more highly expressed in epithelial cells.

Colony stimulating factors (CSFs) varied in expression in each cell type with notable differences in both transcript variants of *Csf1* (Figure IV). This mRNA peaked on days 1 and 4 post mating in the epithelium, while stromal expression remained relatively constant. *Csf2*

showed a minor peak in expression on day 1 post mating in both epithelium and stroma, with epithelial expression peaking on day 4 while stromal expression slowly declined. Of the CSFs, *Csf3* showed the greatest difference in expression between epithelium and stroma, with a dramatic drop in epithelial expression on day 1 followed by a peak on day 2 post mating.

Transforming growth factors (TGFs) were consistently more highly expressed in stroma than in epithelium (Figure V). *Tgfb1* exhibited a divergence between stromal and epithelial expression on day 1 post mating, with stromal expression increasing while epithelial expression decreased. *Tgfb2* showed a similar divergence on day 2 post mating. Interferons (*Ifnb* and *Ifng*) were both detected in murine endometrium, with a higher relative expression noted in stroma (Figure VI). There was one exception to this pattern: *Ifnb* epithelial expression peaked above stromal expression on day 2 *post-coitum*. Differences were noted in other inflammatory cytokines too: *Tnf*, *Lif* and *Ccl5* were all expressed in endometrial stroma at relatively higher levels than in epithelium, with the exception of *Tnf* and *Lif* on day 4 (Figure VII). *Ccl5* expression patterns were similar in both compartments *post-coitum*.

Elements of the prostaglandin pathway were also implicated: prostaglandin synthases were detected in both endometrial epithelium and stroma (Figure VIII). *Ptgs1* (Cox1) demonstrated a dramatic increase in epithelial expression which was relatively higher than stromal expression on day 2 post mating; this was sustained until a day 4 drop. *Ptgs2* (Cox2) peaked in the epithelium on day 2 *post-coitum*. The prostaglandin E receptors *Ptger1*, *Ptger2*, *Ptger3* and *Ptger4* exhibited differences in expression between endometrial epithelial and stromal cells. Both *Ptger1* and *Ptger3* were more highly expressed in stroma than in epithelium. *Ptger1* showed an increase in stromal expression with a concurrent decrease in epithelial expression on day 2 post mating. *Ptger2* and *Ptger4* were consistently more highly expressed in epithelium than in stroma. *Ptger2* peaked on days 1 and 4 post mating, while *Ptger4* peaked on day 3 (Figure VII).

Discussion

This study characterised the murine endometrial epithelial and stromal responses to seminal plasma in the development of maternal immunotolerance to the foetal allograft. Various mediators of this response have been identified. Chief amongst these, CSF2 is secreted into the uterine lumen within hours of *coitus* (Robertson, et al. 1996b, Robertson and Seamark 1990). This ties in well with the present findings, where *Csf2* expression was noted to rise markedly between oestrus and day 1 in both epithelial and stromal compartments. Relative stromal expression exceeded that of the epithelium except on day 4, suggesting that stroma may be the principal source of CSF2. This challenges the conventional epithelium-centric view borne from studies based on trypsinised cell cultures or antibody-based panning techniques which may alter epithelial cell phenotype and response (Robertson, et al. 1992, 1996b, Robertson and Seamark 1990). Moreover, the stromal role is often overlooked in the design of such investigations. *Csf2* mRNA extracted from entire murine uteri post coitum has been shown to be upregulated across days 1-2 (Robertson, et al. 1996a), in contrast to the day 2 decline reported here. This may reflect differences in mouse strain or use of the entire organ mRNA pool rather than the more selective approach used herein. CSF2 was raised on day 1 post mating in both epithelium and stroma, pointing to its likely involvement in the endometrial response to seminal plasma. Epithelial *Csf2* expression was highest on day 4, suggesting its involvement in endometrial receptivity to the implanting blastocyst. Indeed, CSF2 recruits leukocytes to the endometrium to facilitate implantation, with dendritic cells, regulatory T cells and natural killer cells coordinating trophoblast invasion (Bulmer, et al. 1991, Chaouat 2013, Erlebacher 2013, Fu, et al. 2013, Gonzalez, et al. 2012, Hatta, et al. 2012, Hemberger 2013, Male, et al. 2012). Its importance in placentation is highlighted by a study of null mice which showed that absence of CSF2 was associated with late pregnancy loss and delayed foetal growth (Seymour, et al. 1997). CSF1 is a weak chemotactic agent, recruiting macrophages to the epithelium (Wood, et al. 1997), such that its protein levels correlate with macrophage numbers (Pollard, et al. 1987). In the present study, epithelial *Csf1* expression peaked on days 1 and 4, suggesting two waves of macrophage recruitment.

Conceivably, the day 1 wave could be accounted for by the role of macrophages as antigen-presenting cells in the induction of maternal immunotolerance alongside their phagocytic clearance of seminal components within the uterine lumen (Olive, et al. 1987) while on day 4, they may participate in tissue remodelling and increasing epithelial cell adhesiveness in preparation for implantation (Lee, et al. 2011).

Epithelial cluster 1 transcripts (*Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*) peaked on days 1 and 4 post mating. TNFA is key to the acute inflammatory response (Warren, et al. 1988), accounting for its early expression. CXCL3 controls monocyte adhesion and migration (Smith, et al. 2005), thus likely mediating macrophage recruitment. Although CXCL3 is induced by IL13 (Ooi, et al. 2012), their transcripts did not cluster, potentially due to functional redundancy (see later). MMPs also featured prominently in the initial epithelial response to seminal plasma, suggesting some degree of early remodelling and/or neoangiogenic signalling to stroma in preparation for implantation (Rundhaug 2005).

Expression of epithelial clusters 3 (*Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs1*) and 6 (*Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*) was downregulated on day 1 post mating. This is surprising since *Ccl5*, *Csf3*, *Cxcl1* and *Il15* are leukocyte chemotactic/differentiation agents whose levels might be expected to parallel leukocyte activity post coitum (Ashkar, et al. 2003, Bleul, et al. 1996, Kryczek, et al. 2005, Schall, et al. 1990, Wood, et al. 1997). Instead, *Ccl5* and *Il15* expression rose in stroma, suggesting that this compartment may be their principal source. This may also reflect a degree of functional redundancy, a phenomenon partly due to cytokine receptors sharing a common subunit specific to a family of cytokines. For example, IL2 deficiency can be compensated by other cytokines in its family (IL4, IL7, IL9, IL15, IL21) which signal through its receptor by binding to the common γ-chain (Lin, et al. 1995, Ozaki and Leonard 2002). This family of interleukins falls into clusters 3 and 5 whose expression either fails to increase above baseline or is downregulated post mating, with the exception of *Il7* (cluster 2) which peaked on days 1-3. It

is tempting to speculate that *Il7* performs an overarching function on behalf of its family, rendering the others functionally redundant. Alternatively, other mediators may operate as selective suppressors of *Il4*, *Il9*, *Il15* and *Il21* expression although this cannot be determined from our data. The apparent day 1 downregulation of *Il1a* and *Il1b* may be due to the fact that the inflammatory response may peak and fall prior to day 1 sampling. This is conceivable since cytokine expression increases within hours (e.g. <3 hours for *Il1b* in human endometrial epithelial cells *in vitro*) (Gutsche, et al. 2003). It is however more probable that the inflammatory response itself is modified to induce maternal tolerance to paternal antigens without necessarily involving the stereotypical acute phase cytokine cascade.

Prostaglandin-endoperoxide synthases (PTGSs; cyclooxygenases) shape the implantation-related prostaglandin response and influence immune function, angiogenesis and tissue remodelling (Salleh 2014). We found that post coitum *Ptgs1* and *Ptgs2* expression localised to the epithelium, a profile reflected by their protein localisation in both post-mating rats and cycling human uteri (Yang, et al. 2002). Prostaglandin receptor *Ptger1* and *Ptger3* expression rose in stroma in parallel to epithelial *Ptgs2*, suggesting paracrine interactions between the two compartments. Cluster 2 (*Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*) epithelial expression rose post mating and was sustained until day 4. EGF and IL7 are functionally similar in inducing immune effector cell proliferation and differentiation (Niu and Qin 2013) while IL13 modulates human monocyte/B cell function and downregulates/ induces MMPs based on the physiological setting (Bailey, et al. 2012, Firszt, et al. 2013, Wadsworth, et al. 2010). Their sustained mRNA expression supports their role in inducing immunotolerance and stromal remodelling.

As regards the stromal response, cluster 5 (*Ccl5*, *Il1b*, *Inhb1*, *Mcp1*, *Mmp9*, *Thbs1*) was of most interest; expression peaked on days 1-2 post mating and fell on days 3-4. In contrast to the epithelial response, the increase in *Il1b* expression suggests that its stromal function

may relate to its role in governing cell proliferation/differentiation, inducing PTGS2 expression and regulating MMPs (Rossi, et al. 2005), in agreement with the rise in day 2 stromal *Ptgs2* expression. IL1B also likely influences the steroid hormone microenvironment through its suppression of steroid sulphatase activity (Matsuoka, et al. 2002), thereby reducing stromal oestrogenic effects so as to create an environment favourable for nidation. The remaining cluster 5 members mediate leukocyte recruitment and extracellular remodelling in preparation for implantation, as discussed earlier.

Cluster 4 (*Ifng*, *Il15*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*) stromal gene expression levels rose on day 1 and remained high thereafter, in contrast to the epithelium. While these agents regulate epithelial proliferation/remodelling in the menstrual cycle (Godkin and Dore 1998, Gold, et al. 1994), this apparent discrepancy may reflect a paracrine stroma:epithelial relationship, such that bidirectional communication is essential to coordinate the preparatory changes for implantation. TGFB1 can inhibit proliferation and induce PTGS2/prolactin expression in human endometrial stromal cells *in vitro* (Chang, et al. 2008), pointing to a role in tissue remodelling in preparation for decidualisation, as supported by the present data. MMP2 and 9 cleave latent TGFB into its active form (Yu and Stamenkovic 2000), accounting for our observation that *Mmp2* clusters with all TGFB isoforms in both epithelium and stroma. As such, MMP:TGFB signalling pathway interactions may participate in creating the immunopermissive environment of implantation. In contrast with our findings, a murine study showed TGFB2 expression to be confined to the epithelium during the preimplantation period (Das, et al. 1992), which may reflect post-transcriptional processes. *Ifng* and *Tgfb* isoform transcript clustering in stroma was expected given that these cytokines display antagonistic relationships (Eickelberg, et al. 2001, Higashi, et al. 2003, Yu, et al. 2006). In murine and human endometrial epithelium, IFNG has a strong inhibitory effect on TGFB via TGFB type II receptor (*Tgfbr2*) downregulation (Yu, et al. 2006). *Ifng* is unlikely to perform a similar role in stroma since *Tgfbr2* was upregulated on day 1 post mating (data not shown). Instead, the parallel reduction in *Tgfbr2* expression in the epithelium points to paracrine

stromal:epithelial interactions. A likely endometrial role for both these agents revolves around regulating T cell function: IFNG induces T cell apoptosis/inhibits their proliferation, while TGFB promotes naïve T cell differentiation into Tregs (Chen 2011). IFNG also has anti-proliferative effects on human endometrial epithelial cells *in vitro* (Tabibzadeh, et al. 1988) and an inhibitory effect on stromal VEGF production (Kawano, et al. 2000), which is at odds with post coital endometrial events occurring *in vivo*.

To our knowledge, this is the first study to characterise comprehensively the post coitum physiological changes in endometrial epithelial and stromal gene expression. Immune pathways were most affected and exhibited dynamic, quantitatively and chronologically distinct compartment-specific changes. Since the expression and bioactivity of many of the mediators involved are subject to both post-transcriptional and post-translational regulation, future studies should focus analyses at the protein level to draw more functional interpretations. Moreover, the cluster analysis used herein (and in other studies) is based on pre-defined pathways intrinsic to microarray analytical software which may be overly prescriptive and prevent the identification of novel, broader network interactions (e.g. cytokine/eicosanoid/hormone interactions in inflammatory networks). This calls for new analytical and modelling strategies which account for these phenomena.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Legends

Figure I: Schematic diagram representing the complex interplay between murine endometrial epithelial and stromal cells following exposure to semen/seminal plasma. Cytokines, matrix metalloproteinases (MMPs) and prostaglandins (PGs), mediate the development of immunotolerance and tissue remodelling in the peri-implantation period to prepare for implantation and subsequent pregnancy.

Figure II: Cluster analysis of mRNA encoding cytokines and MMPs in murine endometrial epithelial cells. Clusters were identified by a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Cluster membership: Cluster 1 - *Cxcl3*, *Il10*, *Il12a*, *Mmp9*, *Mmp20*, *Mmp25*, *Tnf*; Cluster 2 - *Bmp4*, *Egf*, *Il7*, *Il13*, *Mmp1a*, *Mmp3*, *Mmp16*, *Mmp28*; Cluster 3 - *Ccl5*, *Csf3*, *Il1b*, *Il15*, *Inhb1*, *Mmp11*, *Mmp14*, *Mmp23*, *Thbs*; Cluster 4 - *Ifng*, *Mcp1*, *Mmp2*, *Mmp19*, *Tgfb1*, *Tgfb2*, *Tgfb3*; Cluster 5 - *Csf2*, *Cxcl2*, *Il2*, *Il4*, *Il5*, *Il12b*, *Itbp1*, *Mmp10*, *Mmp12*, *Mmp13*, *Mmp21*, *Mmp24*; Cluster 6 - *Cxcl1*, *Il1a*, *Lif*, *Mmp8*, *Mmp27*; Cluster 7 - *Csf1t1*, *Csf1t3*, *Mmp7*; Cluster 8 - *Ifnb*, *Il6*, *Il11*.

Figure III: Cluster analysis of mRNA encoding cytokines and MMPs in murine endometrial stromal cells. Clusters were identified by a two-step cluster analysis using Ward linkage. At each iteration, the Δ coefficient was calculated, with the largest Δ coefficient defining the number of clusters. Cluster membership: Cluster 1 - *Csf1t1, Csf3, Mmp23;* Cluster 2 - *Bmp4, Csf1t3, Csf2, Cxcl2, Cxcl3, Egf, Ifnb1, Il1a, Il2, Il4, Il5, Il6, Il7, Il10, Il11, Il12a, Il12b, Il13, Mmp1a, Mmp3, Mmp8, Mmp10, Mmp13, Mmp12, Mmp16, Mmp20, Mmp21, Mmp24, Mmp25, Mmp28, Tnf;* Cluster 3 - *Mmp7;* Cluster 4 - *Ifng, Il15, Mmp2, Mmp19, Tgfb1, Tgfb2, Tgfb3;* Cluster 5 - *Ccl5, Inhb1, Il1b, Mcp1, Mmp9, Thbs1;* Cluster 6 - *Mmp11, Mmp14;* Cluster 7 - *Cxcl1, Itbp1, Lif, Mmp27.*

Figure IV: Relative CSF mRNA expression in murine endometrial epithelial and stromal cells post coitum. *Csf1* (both transcript variants) varied little in the epithelium, while peaking on day 1 and 4 in the stroma. *Csf2* exhibited higher stromal expression at all time points except day 4. *Csf3* exhibited the most variation across cell type and time point.

Figure V: Murine endometrial cell relative TGFB mRNA expression in the pre-implantation period. All *Tgfb* isoforms were consistently more highly expressed in the stromal compartment post mating. With the exception of *Tgfb1* which demonstrated a reduction in stromal compartment expression, all isoforms exhibited a rise in expression in day 1 post mating.

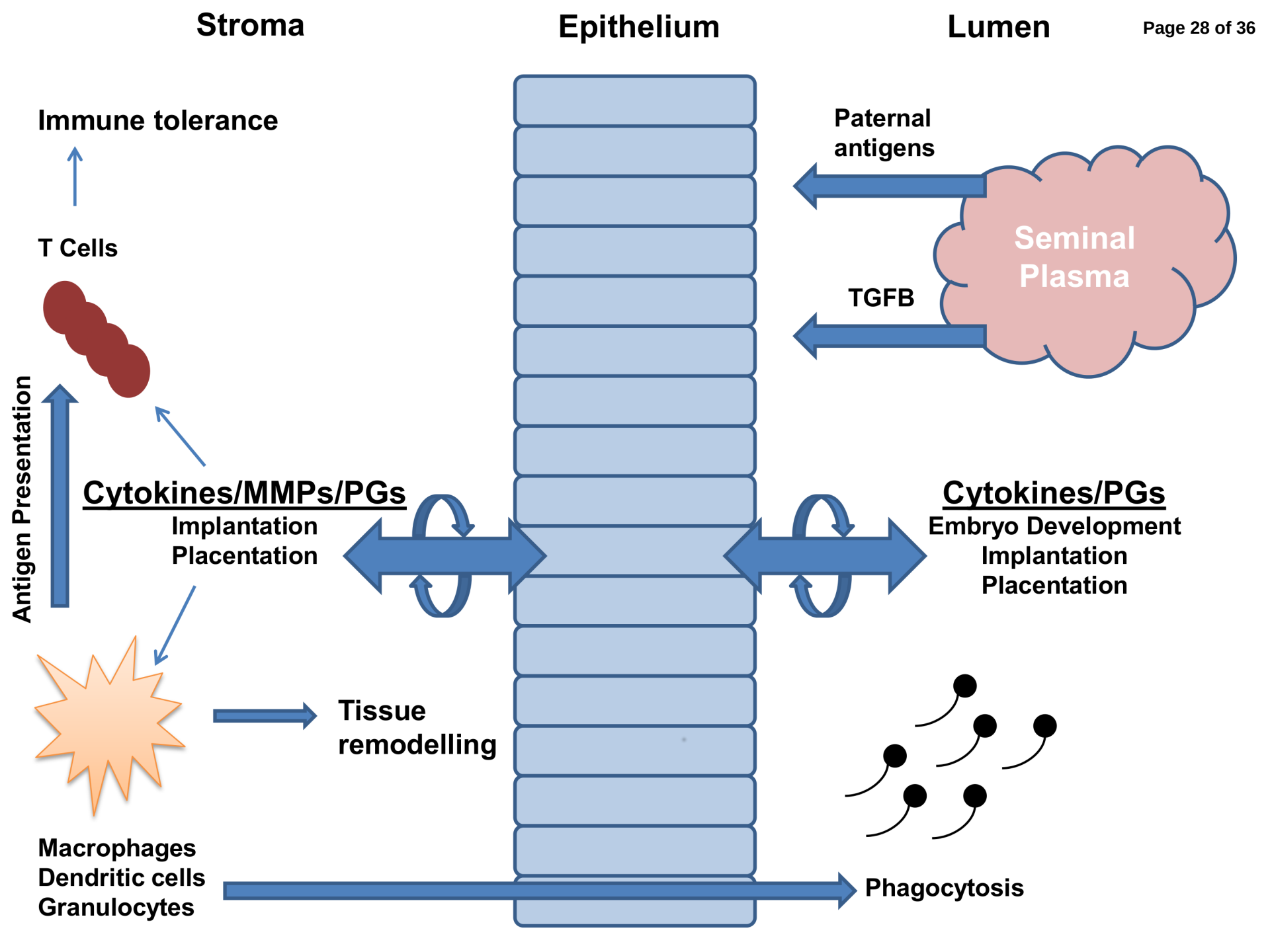
Figure VI: Relative *Ifnb* and *Ifng* expression in murine endometrial cell compartments post mating. *Ifnb* exhibited a sharp peak in expression on day 2 post mating in the stromal compartment, while *Ifng* showed a peak at this time point in the epithelium.

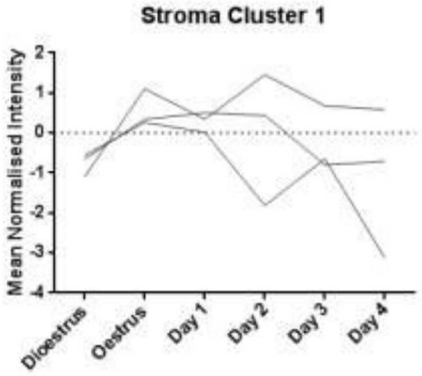
Figure VII: Relative expression (mean normalised intensity) of *Lif*, *Tnf* and *Ccl5* in murine endometrial epithelial and stromal compartments. *Lif* expression remained relatively constant excepting at Day 4, while *Tnf* expression profiles were similar in both compartments although with higher expression in the stromal compartment at all days except Day 4. *Ccl5* expression was consistently more highly expressed in the stromal compartment.

Figure VIII: Murine endometrial expression of prostaglandin pathway elements during the pre-implantation period. Synthases *Ptgs1* and *Ptgs2* peaked on day 2 in the epithelium with a subsequent decline to day 4. Receptor expression varied, with *Ptger1* and *Ptger3* having relatively higher stromal expression while *Ptger2* and *Ptger4* followed the opposite pattern.

Supplementary Figure I: Example of laser capture microdissection of murine endometrial epithelial and stromal compartments. Epithelial and stromal compartments were identified

and excised. Areas pre- and post-excision are highlighted in green (top panel epithelial compartment, bottom panel stromal compartment).


Stroma
Epithelium
Lumen
Immune tolerance
T Cells
Antigen Presentation
Paternal antigens
Seminal Plasma
TGFB
Cytokines/MMPs/PGs
Implantation
Placentation
Cytokines/PGs
Embryo Development
Implantation
Placentation
Tissue remodelling
Macrophages
Dendritic cells
Granulocytes
Phagocytosis


Stroma Cluster 1
Mean Normalised Intensity
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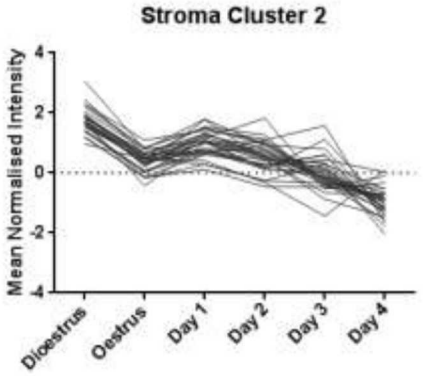
-1
-2
-3
-4
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4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0
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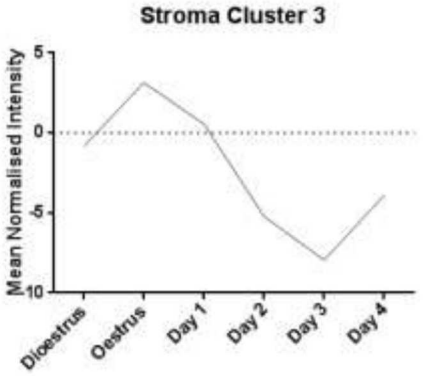
-4
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4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Mean Normalised Intensity
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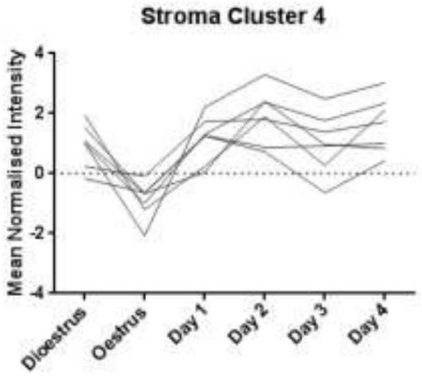
-10
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4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Mean Normalised Intensity
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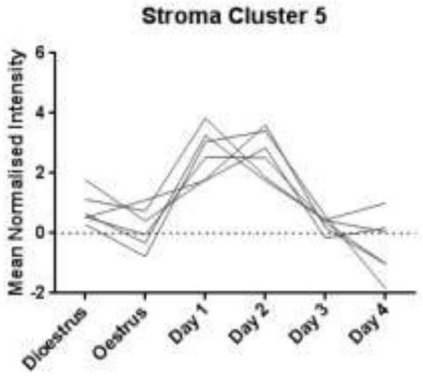
-2
-4
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4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Mean Normalised Intensity
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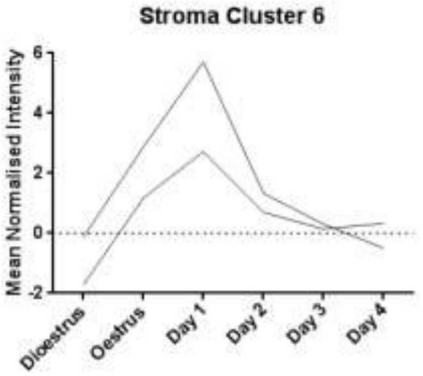
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4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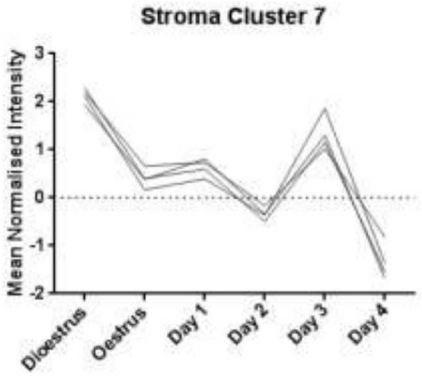
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Stroma Cluster 7
Mean Normalised Intensity
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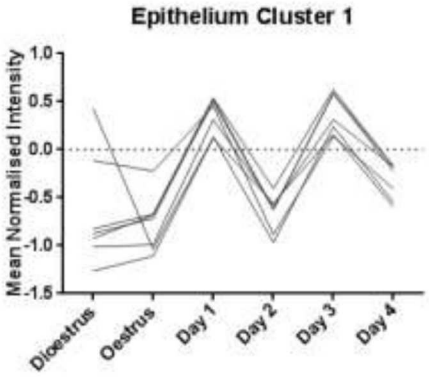
2
1
0
-1
-2
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Epithelium Cluster 1
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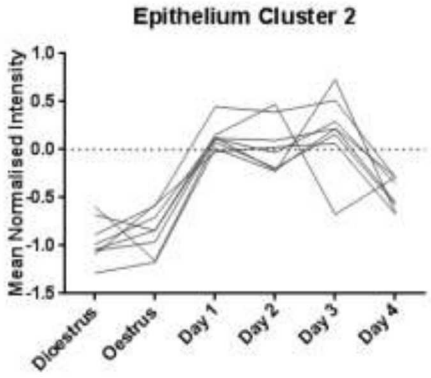
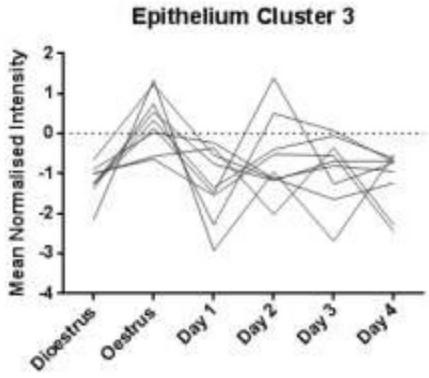
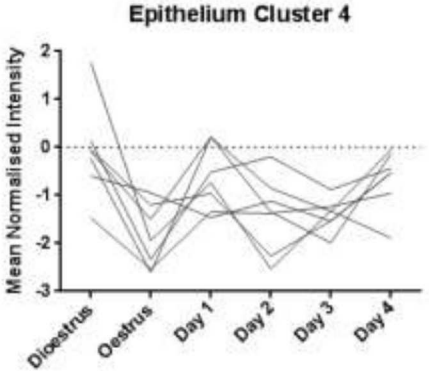
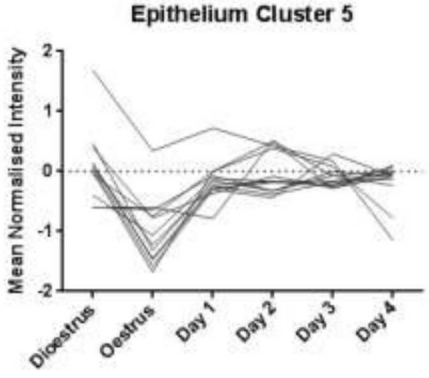
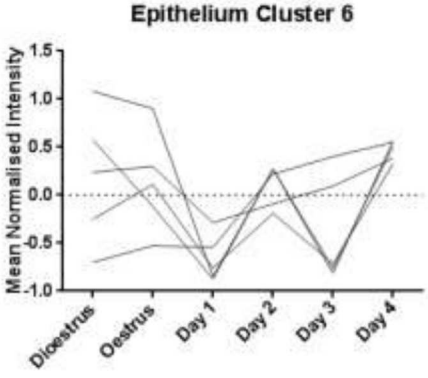
-1.5
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

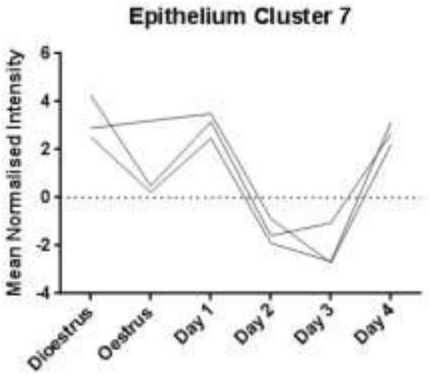

Epithelium Cluster 2
Mean Normalised Intensity
1.0
0.5
0.0
-0.5
-1.0
-1.5
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

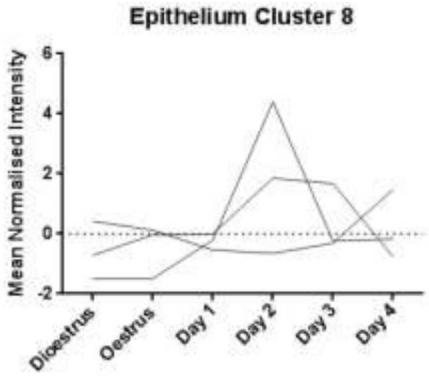

Epithelium Cluster 3
Mean Normalised Intensity
2
1
0
-1
-2
-3
-4
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

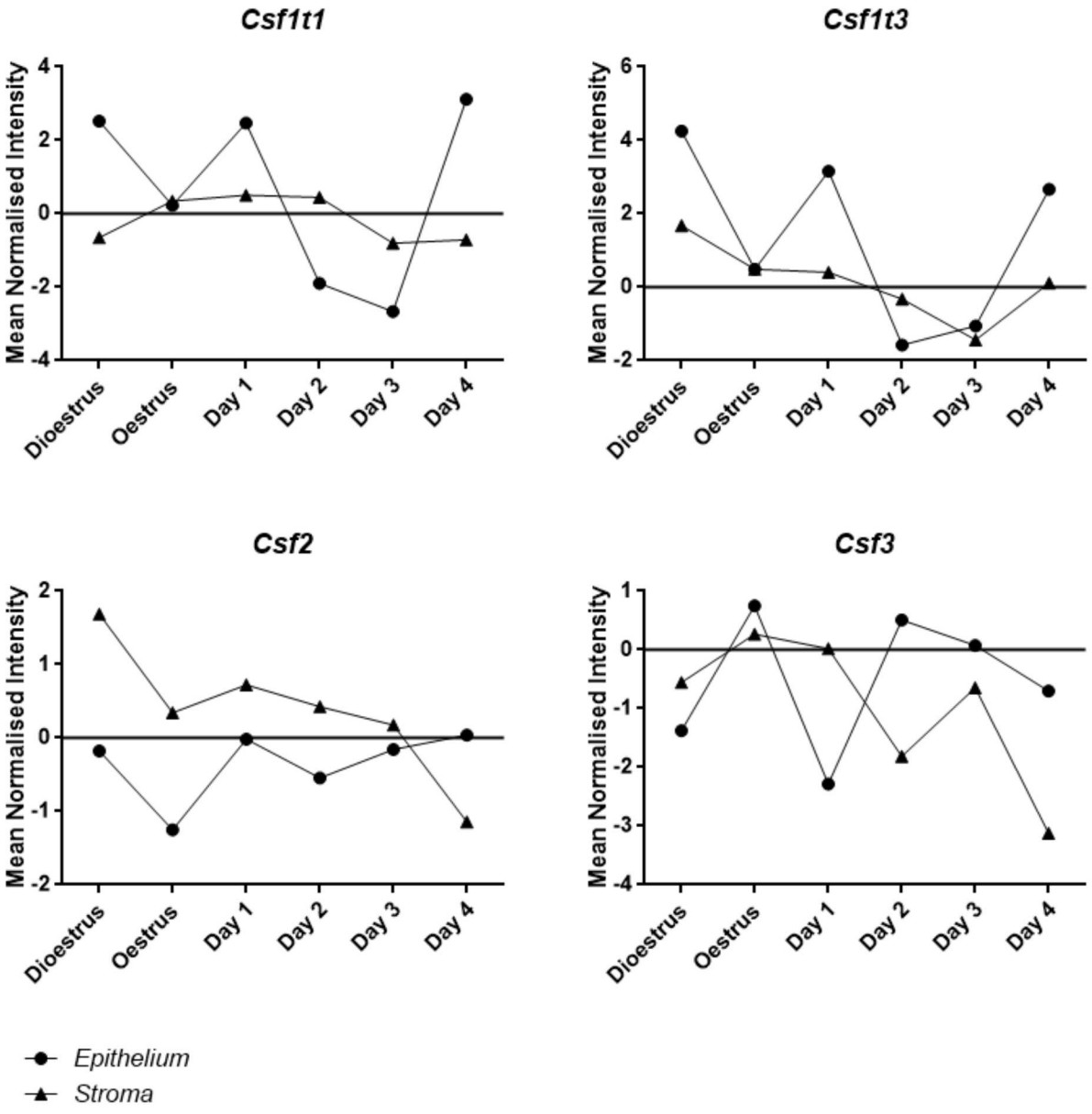

Epithelium Cluster 4
Mean Normalised Intensity
2
1
0
-1
-2
-3
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

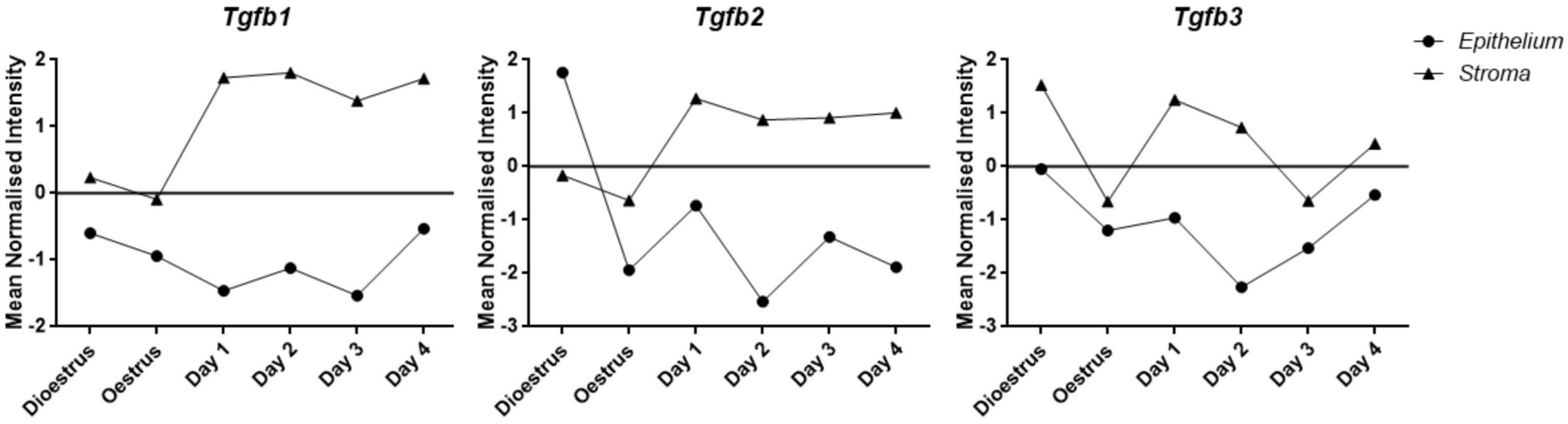

Epithelium Cluster 5
Mean Normalised Intensity
2
1
0
-1
-2
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

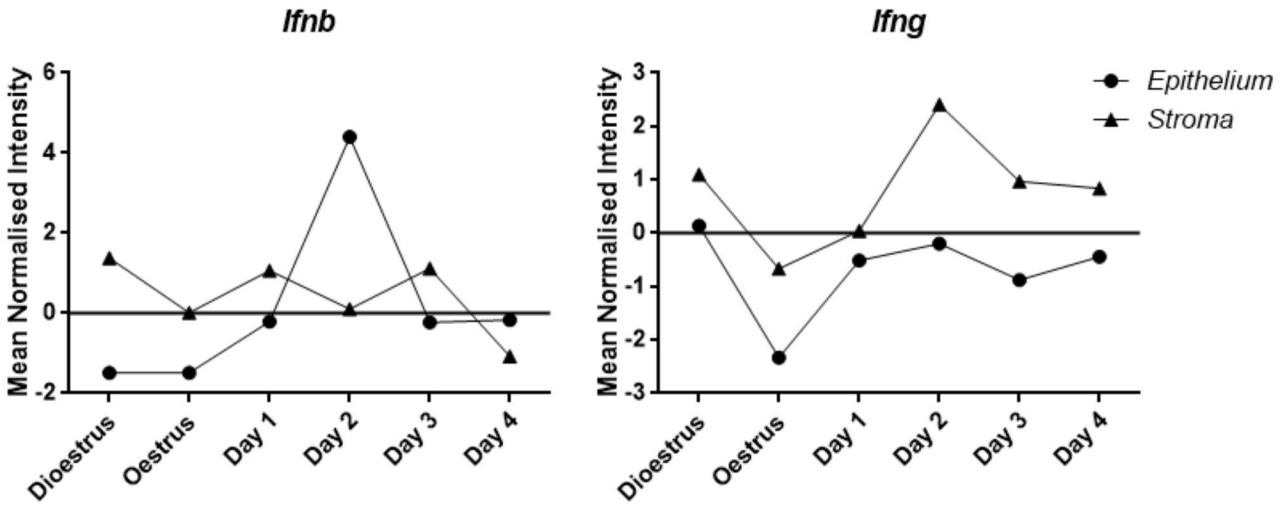

Epithelium Cluster 6
Mean Normalised Intensity
1.5
1.0
0.5
0.0
-0.5
-1.0
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

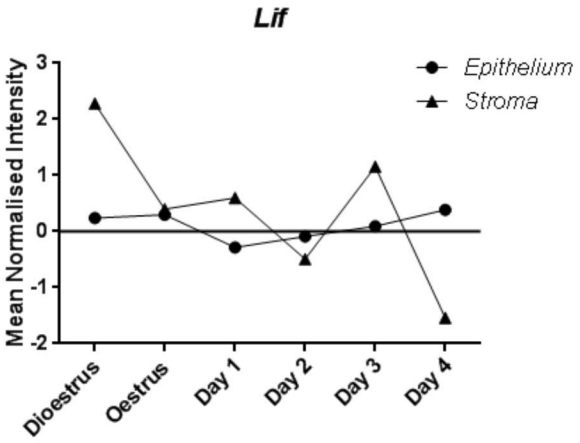

Epithelium Cluster 7
Mean Normalised Intensity
6
4
2
0
-2
-4
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

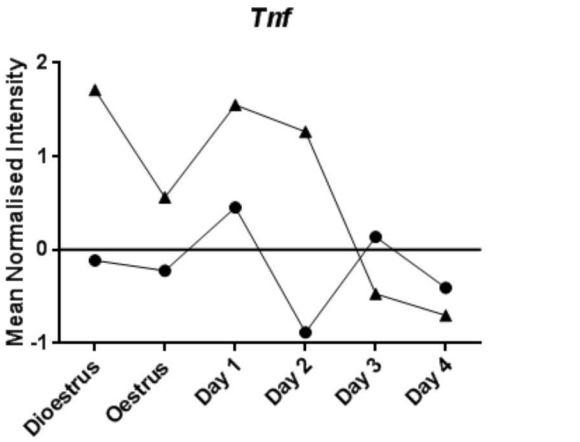

Epithelium Cluster 8
Mean Normalised Intensity
6
4
2
0
-2
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

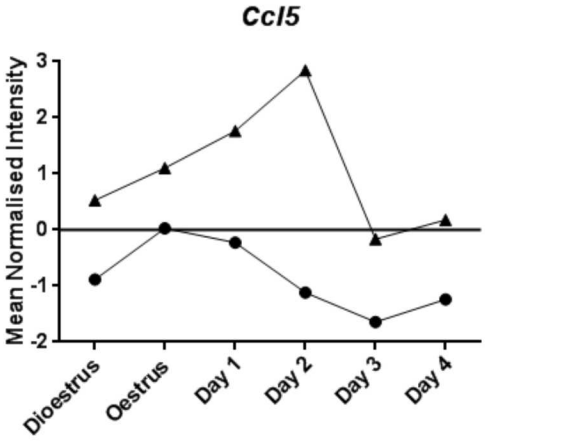

Csf1t1
Csf1t3
Csf2
Csf3
Mean Normalised Intensity
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4
Epithelium
Stroma


Tgfb1
Tgfb2
Tgfb3
Epithelium
Stroma
Mean Normalised Intensity
2
1
0
-1
-2
-3
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Ifnb
Ifng
Mean Normalised Intensity
6
4
2
0
-2
3
2
1
0
-1
-2
-3
Epithelium
Stroma
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Lif
Epithelium
Stroma
Mean Normalised Intensity
3
2
1
0
-1
-2
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Tnf
Mean Normalised Intensity
2
1
0
-1
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4


Ccl5
Mean Normalised Intensity
3
2
1
0
-1
-2
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

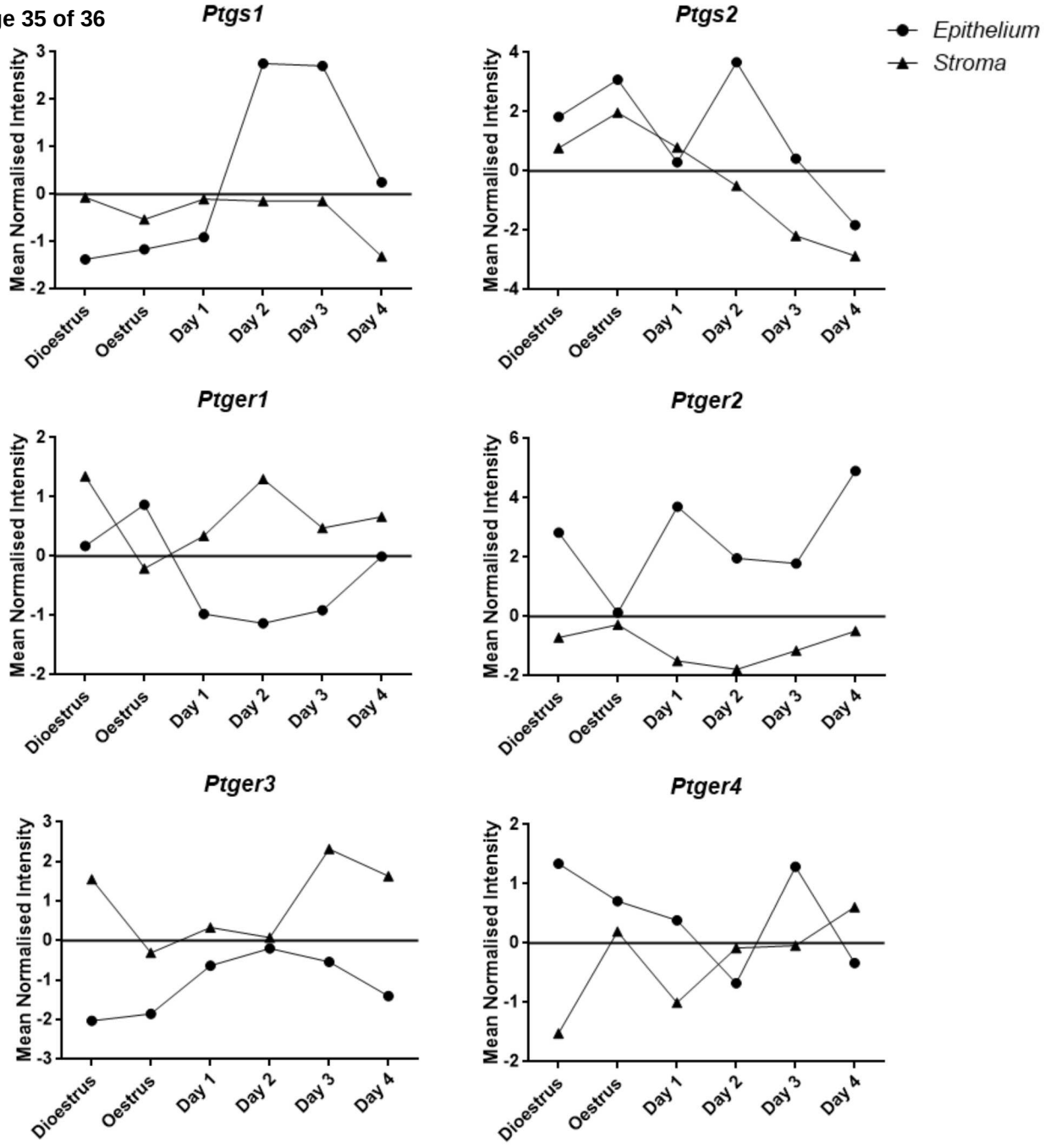

Ptgs1
Ptgs2
Epithelium
Stroma
Ptger1
Ptger2
Ptger3
Ptger4
Mean Normalised Intensity
Dioestrus
Oestrus
Day 1
Day 2
Day 3
Day 4

Table I: Pathways active in murine endometrial epithelial and stromal cells.

Pathway	WikiPathways ID	p-value	Number of Matched Entities	Number of Pathway Entities
GPCRs, Class A Rhodopsin-like	WP189 62834	0.00	208	231
GPCRs, Other	WP41 54691	0.00	150	210
GPCRs, Other	WP41 62667	0.00	150	210
Cytoplasmic Ribosomal Proteins	WP163 62833	0.00	77	80
Peptide GPCRs	WP234 41308	0.00	63	70
Metapathway biotransformation	WP1251 41349	0.00	117	143
Monoamine GPCRs	WP570 48232	0.00	32	33
Monoamine GPCRs	WP570 60231	0.00	32	33
Calcium Regulation in the Cardiac Cell	WP553 47774	0.00	120	150
Striated Muscle Contraction	WP216 41273	0.00	40	45
Complement and Coagulation Cascades	WP449 41301	0.00	53	62
Prostaglandin Synthesis and Regulation	WP374 41394	0.00	29	31
Small Ligand GPCRs	WP353 41279	0.00	18	18
Selenium	WP1272 58514	0.00	22	31
Cytokines and Inflammatory Response (BioCarta)	WP222 53571	0.00	26	30
Selenium	WP1272 59028	0.00	21	31
Complement Activation, Classical Pathway	WP200 47967	0.00	16	17
Cytochrome P450	WP1274 48227	0.00	35	40
Myometrial Relaxation and Contraction Pathways	WP385 47969	0.00	120	157
Blood Clotting Cascade	WP460 62696	0.00	19	20
Glucocorticoid & Mineralcorticoid Metabolism	WP495 62838	0.00	13	13
SIDS Susceptibility Pathways	WP1266 58254	0.00	49	61
SIDS Susceptibility Pathways	WP1266_58281	0.00	49	61
GPCRs, Class B Secretin-like	WP456 41317	0.01	20	22
Nuclear receptors in lipid metabolism and toxicity	WP431 47744	0.01	26	30
Glucuronidation	WP1241 59029	0.01	11	18
Irinotecan Pathway	WP475 48258	0.01	11	11
Matrix Metalloproteinases	WP441 41300	0.01	25	29
Macrophage markers	WP2271 53132	0.01	10	10
XPodNet - protein-protein interactions in the podocyte expanded by STRING	WP2309 58142	0.01	573	836
XPodNet - protein-protein interactions in the podocyte expanded by STRING	WP2309 58143	0.01	573	836
Oxidative Stress	WP412 41381	0.02	24	29
GPCRs, Class C Metabotropic glutamate, pheromone	WP327 41361	0.02	14	15
Folic Acid Network	WP1273 48256	0.02	20	27
Inflammatory Response Pathway	WP458 57463	0.03	25	30
TGFβ Signaling Pathway	WP113 41270	0.03	41	52
Metapathway biotransformation	WP1251 59060	0.03	22	143
Hedgehog Signaling Pathway	WP116 41332	0.03	19	22
Folic Acid Network	WP1273 60224	0.03	19	27
Glycolysis and Gluconeogenesis	WP157 43573	0.03	38	48
Endochondral Ossification	WP1270 41292	0.03	48	62
Serotonin and anxiety-related events	WP2140 58159	0.03	12	13
Glycolysis and Gluconeogenesis	WP157 51735	0.04	39	50
ESC Pluripotency Pathways	WP339 42897	0.04	81	111
Retinol metabolism	WP1259 51364	0.04	31	39
Oestrogen metabolism	WP1264 48250	0.04	11	13