JCI insight

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JCI Insight. 2022. https://doi.org/10.1172/jci.insight.135407.

Research In-Press Preview **Immunology** **Reproductive biology**

Although published studies have demonstrated that interferon epsilon (IFNε) has a crucial role in regulating protective immunity in the mouse female reproductive tract (mFRT), expression and regulation of IFNε in the human female reproductive tract (hFRT) have not been characterised. To characterise human IFNε, we obtained hFRT samples from a well- characterized cohort of women, enabling us to comprehensively assess ex vivo IFNε expression in the hFRT at various stages of the menstrual cycle. We found that among the various types of IFNs, IFNε is uniquely selectively and constitutively expressed in the hFRT epithelium. It has distinct expression patterns in the surface and glandular epithelia of the upper hFRT compared with basal layers of the stratified squamous epithelia of the lower hFRT. There is cyclical variation of IFNε expression in the endometrial epithelium of the upper hFRT and not in the distal FRT, consistent with selective endometrial expression of the progesterone receptor and regulation of the IFNE promoter by progesterone. Since we show IFNε stimulates important protective IFN-regulated genes (IRGs) in FRT epithelium, this characterisation is a key element in understanding the mechanisms of hormonal control of mucosal immunity.

Spatiotemporal regulation of human IFNε and innate immunity in the female reproductive tract

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Conflict of Interest Statement

SLA has received consulting fees from Mayne Pharma and Merck. Magee-Womens Research Institute receives research funding from Estetra SRL, EvoFem, and Merck. The other authors have declared that no conflict of interest exists.

Abstract

Although published studies have demonstrated that interferon epsilon (IFNε) has a crucial role in regulating protective immunity in the mouse female reproductive tract (mFRT), expression and regulation of IFNε in the human female reproductive tract (hFRT) have not been characterised. To characterise human IFNε, we obtained hFRT samples from a well-characterized cohort of women, enabling us to comprehensively assess ex vivo IFNε expression in the hFRT at various stages of the menstrual cycle. We found that among the various types of IFNs, IFNε is uniquely selectively and constitutively expressed in the hFRT epithelium. It has distinct expression patterns in the surface and glandular epithelia of the upper hFRT compared with basal layers of the stratified squamous epithelia of the lower hFRT. There is cyclical variation of IFNε expression in the endometrial epithelium of the upper hFRT and not in the distal FRT, consistent with selective endometrial expression of the progesterone receptor and regulation of the *IFNE* promoter by progesterone. Since we show IFNε stimulates important protective IFN-regulated genes (IRGs) in FRT epithelium, this characterisation is a key element in understanding the mechanisms of hormonal control of mucosal immunity.

Introduction

The human female reproductive tract (hFRT) mucosa is a unique site of immune regulation requiring robust responses against pathogenic infections yet maintaining tolerance with commensal bacteria, semen, and developing pregnancies (1). Epithelial cells lining the vagina, cervix and uterus form an initial barrier against invading pathogens and are important regulators of immunity with specialised capabilities, including antigen presentation and secretion of mucins, antimicrobial peptides (AMPs), and chemokines that modulate recruitment and activation of the innate and adaptive immune cells (2). The fluctuation of sex hormones in women, primarily estradiol (E2) and progesterone, across the menstrual cycle may impact immune function in the hFRT, although the mechanisms mediating these effects are not comprehensively defined (3). During the progesterone-dominated luteal phase of menstrual cycle when the endometrium prepares for fertilisation and implantation, immune responses are relatively suppressed and a tolerogenic state is established (3). However, this environment may also be more permissible to pathogen invasion. For example, depot medroxyprogesterone acetate is commonly used in animal models, including human immunodeficiency virus (HIV) animal models, to induce higher susceptibility to *Chlamydia trachomatis* and HSV2 infections (4, 5). Factors that may mediate hormonal effects on host defence in the hFRT are not known. Antiviral and immunoregulatory cytokines, such as type I interferon (IFNs), are prime candidates (6).

The type I IFNs are a family of cytokines that include the conventional α and β subtypes, as well as the more recently identified IFNε. All type I IFNs bind IFN-α receptor 1 (IFNAR1) and IFNAR2, activate JAK-STAT signalling and regulate the expression of potentially thousands of IFN-regulated genes (IRGs) (7). The ‘effector’ proteins encoded by IRGs can modulate a wide range of biological responses, including antiviral activities, cell cycle regulation,

survival/apoptosis, immune effector cell activity and chemotaxis. Epithelial cells that line the vaginal, cervical and endometrial mucosa are key sentinels that produce conventional type I IFNs (e.g. α's and β) upon pathogen challenge (2). IFNε, which we characterised in mouse female reproductive tract (mFRT) to be constitutively expressed in the endometrium and protective against viral and bacterial STIs (8), may have an important role in STI protection in the mFRT. We determined that murine IFNε was not induced by pattern recognition receptor (PRR) pathways like conventional type I IFNs, interestingly, we found that IFNε was hormonally regulated and IFNε levels fluctuated in the endometrium across the estrus cycle. Furthermore, in vitro experiments conducted with human cells demonstrated that IFNε can block HIV replication at several steps of viral replication through induction of antiviral IRGs (9, 10). The expression, distribution, regulation, and functions of IFNε in the hFRT are currently unknown. Therefore, we designed and conducted a cross-sectional study of healthy reproductive-aged women with normal menstrual cycles and aimed to characterize IFNε expression in the hFRT during the follicular and luteal phases of menses.

Results

Participant enrollment and demographic characteristics

The 33 enrolled eligible participants in cohort 1 were allocated to follicular phase or luteal phase analysis groups based on serum progesterone concentration measured by ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS). We defined follicular and luteal phases of menses as serum progesterone <1000 pg/mL and >2000 pg/mL respectively. The demographic characteristics did not differ by allocated menstrual phase arm (Table 1). The median serum progesterone concentrations were 49 (IQR 39.5, 318.5) and 6171 (IQR 2998, 9774) pg/mL for participants in the follicular and luteal analysis groups respectively.

IFNε is expressed throughout the hFRT

Immunohistochemical (IHC) evaluation of matched vaginal, ectocervical, and endometrial biopsy samples from all participants in cohort 1 demonstrated that IFNε was highly expressed in the stratified squamous epithelium localised to the basal and parabasal layers of the lower hFRT (vagina and the ectocervix) (Figure 1B and C). In the endometrium, strong IFNε staining was detected in both the luminal and glandular epithelium (Figure 1A). Thus IFNε is constitutively expressed appropriately to exert physiological functions locally throughout the hFRT.

Selective cyclic variation of endometrial IFNε

Examination of IFNε expression in the endometrium at the spatial and molecular level in cohort 1 revealed that there was significantly higher *IFNE* mRNA abundance in the endometrium

during the luteal phase of menstrual cycle compared with expression during the follicular phase ($p<0.001$; Figure 2A). Similarly, there was significantly higher IFNε protein in the epithelium of the endometrium during the luteal phase of cycle compared with the follicular phase ($p<0.05$; Figure 2B and 2C). There were no differences in vaginal and ectocervical *IFNE* mRNA (Figure 2A) or IFNε protein by menstrual cycle phase (Supplemental Figure 1A and 1B).

IFNε expression is negatively regulated by progesterone receptor

Since variation of IFNε expression in the menstrual cycle occurred only in the upper hFRT, we examined the expression patterns of hormone receptors that might mediate this differential regulation in this cohort. We found high expression of progesterone receptor (PR) transcript (Figure 3A) in the endometrium compared with very low expression of PR transcript in the ectocervix and vagina. Accordingly, IHC analysis of PR protein (Figure 3B) across the hFRT revealed strong staining of PR in both luminal and glandular endometrial epithelial cells, but it was barely detectable in the ectocervix and vagina. Although *PGR* mRNA was not evidently different in the endometrium based on cycle stage (Figure 3C), endometrial PR protein was significantly lower during the luteal stage of the menstrual cycle (Figure 3D and 3E, $p<0.01$). These changes were particularly evident in the epithelial cytoplasmic PR abundance

Regression analysis of our independent measures of PR and IFNε expression levels for individual samples showed an inverse correlation that was statistically significant at both the mRNA and protein level ($p<0.01$; Figure 4A and B respectively). We found no correlation between *IFNE* and *ESR1* (Supplemental Figure 2A and 2B).

To investigate whether this inverse correlation was due to a direct suppression of IFNε expression by PR, we used an endometrial epithelial cell line, ECC-1, to characterise the

regulation by progesterone of a luciferase gene construct under the control of the human *IFNE* promoter. In this in vitro system, progesterone stimulation significantly inhibited activation of the human *IFNE* promoter ($p<0.001$; Figure 4B) and estrogen stimulation had no effect on promoter activity. To confirm this finding in primary uterine epithelial cells, we cultured cells from endometrial biopsies obtained from cohort 2, stimulated them with progesterone or estradiol and assessed *IFNE* expression. In this ex vivo model, *IFNE* expression significantly decreased following 3 hours of in vitro stimulation with progesterone ($p<0.05$; Figure 4C) whereas in vitro stimulation with estrogen did not alter *IFNE* expression, despite these cells being responsive to estrogen stimulation (Supplemental Figure 3C). These data demonstrated a negative regulation of human IFNε expression by progesterone.

IFNε regulates protective immunoregulatory pathways in the hFRT

In order to determine whether this constitutive, hormone regulated IFNε was active in modulating immune responses in the human FRT, we measured the expression of IRGs that encode known immune effector and signaling proteins in cohort 1 samples. All samples collected, regardless of phase of menses or sample type (vaginal, cervical, or endometrial), demonstrated strong and significant correlation of *IFNE* mRNA expression with expression of antiviral IRGs (*MX1 OAS2*, *IRF7),* chemokines (*CXCL10)*, pathogen sensing IRGs (*DDX58),* and IFN signaling (*STAT1*) (Figure 5A and Supplemental Figure 3A).

To confirm that IFNε directly induces innate immune effector molecules, we stimulated vaginal epithelial cells (VK2 cell line), ectocervical epithelial cells (Ect1 cell line) (Supplemental Figure 3B) and uterine epithelial cells (primary cells cultured from endometrial biopsies from cohort 2, Figure 5B) with exogenous IFNε. The IRGs *MX1, OAS2,* and *CXCL10* were substantially induced around 10-fold to 100-fold respectively.

IFNε is the sole IFN expressed constitutively in the hFRT.

While these data are consistent with the hypothesis that basal expression of IFNε in the FRT constitutively maintains innate immune responses at this site, many IFNs could regulate these types of responses. Until now, however, the relative expression of all IFNs in the FRT has not been comprehensive and conclusively examined. This study reveals that *IFNE* was selectively and strongly expressed in the vagina, ectocervix and endometrium relative to expression of other type 1 (*IFNA1*, *IFNA2*, *IFNA4*, *IFNB*), type II (*IFNG*) and type III (*IFNL1*, *IFNL2* and *IFNL3*) IFNs ($p<0.0001$), each of which was either undetectable or had very low expression compared to *IFNE* expression (Figure 5C).

Therefore, these data constitute a compelling case that IFNε is the predominant driver of IFN-dependent immunity in homeostatic conditions in the FRT based on the observations that: 1) IFNε expression levels correlate significantly with induction of important immunoregulatory genes; 2) these genes were independently demonstrated to be inducible by direct action of IFNε on FRT epithelial cells; and 3) IFNε is essentially the only IFN expressed across the FRT.

IFNε protein expression is detectable in cervicovaginal lavage fluid

We developed a sandwich ELISA using in-house monoclonal antibodies, which detected significant levels of IFNε production in matched cervicovaginal lavage (CVL) samples collected from cohort 1 confirming IFNε protein expression in FRT secretions (Figure 6A). We compared levels of IFNε to two other cytokines known to be expressed in CVL – IL-15 and IL-6. IFNε levels in CVL were similar to IL-15 levels (0-20 pg/ml); IL-6 expression was notably higher than both of these cytokines (Figure 6A). Interestingly, we did not find that IFNε expression in CVLs differed based on menstrual cycle stage, similar to IL-6 and unlike

IL-15 which was higher in CVLs from women in luteal stage of cycle compared to follicular (Figure 6B). This was despite evident hormonally regulation of *IFNE*, *IL15* and *IL6* mRNA in endometrial biopsy samples where expression of all three transcripts were significantly higher in luteal stage of cycle (Figure 6C). Similar to *IFNE*, neither *IL15* or *IL6* mRNA expression in the ectocervix or vagina differed significantly based on cycle stage. Endometrial *IL15* mRNA expression did correlate with IL-15 levels in CVL (Spearman r 0.44, p=0.01) yet endometrial *IFNE* or *IL6* mRNA expression did not correlate with levels of these cytokines in CVL. Furthermore, the epithelial origin of IFNε in the FRT is confirmed by its detection in lysates of cultured FRT derived cancer cell lines (data not shown).

Discussion

This study provides characterisation of the spatiotemporal expression and hormonal regulation of IFNε in distinct parts of the hFRT from a well-characterised cohort of 33 women. Importantly, the study design enabled evaluation of IFNε expression throughout the hFRT and these data further our previous mechanistic studies conducted in mouse models. Here we demonstrate that IFNε expression (assessed by mRNA and protein abundance) is contiguously expressed from the lower to the upper hFRT at major sites requiring immune protection and is also detectable in hFRT secretions. IFNε is highly expressed in the luminal and glandular epithelium of the human endometrium, the site of implantation and of immune importance for protection against ascending infections such as *Chlamydia*. There was also stronger expression of IFNε in the basal layers of the stratified squamous epithelium of the cervix and vagina, which are important sites of infection with viruses such as HIV, whose replication we and others have demonstrated can inhibited by IFNε (9) (10).

A clear finding in this study was that *IFNE* was the sole IFN (type I, II or III) measured that was substantially, consistently and constitutively expressed throughout the hFRT. IFNε expression in cervicovaginal lavage samples was similar to the concentrations of IL-15 detected in this sample type. IFNε is therefore a prime candidate for regulating homeostatic signals to fine tune the innate immune system in the hFRT mucosal surfaces. The efficacy of IFNε regulation of homeostatic immunity was the demonstration that IFNε can induce IRGs with important functions. We further demonstrated that immunoregulatory IRG expression in hFRT samples was strongly correlated with the high *IFNE* expression in the hFRT. These data agree with our previously reported mouse model data study showing that IFNε$^{-/-}$ mice have reduced IRG expression in the mFRT (8). 'Basal' constitutive expression of regulatory IRGs that can modulate cellular processes such as metabolism, differentiation, proliferation, survival, angiogenesis, etc. – in addition to their more prominent protective role in viral and bacterial infection and general immunoregulation – may be a crucial role for this unique IFN in tonic signaling to tune the mucosal innate immune system.

The data herein demonstrate that progesterone receptor (*PGR*) is predominantly expressed in the endometrium and that *IFNE* expression is suppressed by progesterone, suggesting that IFNε production is hormonally regulated in the hFRT. Surprisingly, this regulation appears to occur in the endometrium and not in the vagina or ectocervix. This is likely because *PGR* expression in high in endometrial cells compared with cells in the vagina and ectocervix. Indeed, there is a substantial and significant inverse correlation between IFNε and progesterone receptor expression at the mRNA and protein level across all individuals in this study. Progesterone receptor expression is itself regulated by estradiol and future studies are warranted to decipher the complex relationships between estrogen responses, progesterone receptor regulation and IFNε expression in endometrial epithelial cells. While we did not observe hormonal regulation in the lower hFRT, *IFNE* expression has been previously demonstrated to be upregulated in

the human ectocervix upon exposure to semen (11, 12) and has been hypothesized to have an immunomodulatory mechanism within the hFRT to reduce risk of acquisition of HIV (13).

Strengths and limitations: A major strength of this work was that the data generated on ex vivo IFNε expression and associated hormonal responses (cohort 1), with the exception of the cell line and primary uterine epithelial cell work (cohort 2), were taken from a tightly controlled clinical study with strict inclusion and exclusion criteria to ensure we could account for many confounding factors, such as infections, medications, exogenous hormonal influences etc, that many other studies based on FRT samples have not done. Each participant had matched samples taken from the FRT at time of enrolment, including matched vaginal, cervical and endometrial biopsy samples, as well as cervical lavages. We performed MS analysis to confirm cycle stage rather than rely solely on self-reported and/or histological approaches. For all IHC analysis, we created tissue arrays with each slide containing matched vaginal, cervical and endometrial tissue sections from up to nine participants in our study, thus ensuring that all IHC staining was carefully controlled to minimise inter- and intra- individual variation. We revealed the relationship between IFNε abundance in the endometrium and progesterone responses, but further work is required to conclusively investigate the specific mechanisms of this relationship and what this may mean for women on hormone-based contraceptives.

Conclusion: IFNε likely has an important defence role in the hFRT against pathogens, since it is constitutively expressed in the ectocervix and throughout the hFRT and regulates immune modulation in hFRT epithelium. Our results demonstrating IFNε suppression via the progesterone receptor warrant further study to evaluate if use of hormonal contraceptives with high affinity for the progesterone receptor may also suppress IFNε and whether this may impact susceptibility to sexually transmitted infections, including HIV.

Methods

Participant recruitment and sampling:

Cohort 1: We performed a cross-sectional study (ClinicalTrials.gov number: NCT02416154) at the Magee-Womens Research Institute, Pittsburgh, PA, USA of healthy, reproductive-aged women with normal menstrual cycles who were free of exogenous hormonal contraceptives. The University of Pittsburgh Institutional Review Board and the Monash Health Human Research Ethics Committee both approved this study. All participants were enrolled at the Centre for Family Planning Research at Magee-Womens Hospital in Pittsburgh, Pennsylvania and signed informed consent before study participation.

Being free of exogenous steroid hormones and in a defined (follicular vs luteal) phase of menses was central to the study design and therefore laboratory confirmation by ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) was performed to evaluate serum progesterone and estradiol, as well as a panel of synthetic progestins that cover the majority of regionally available contraceptive progestins.

Between August 2015 and August 2016, 44 participants were assessed for study eligibility and 34 women, age 18–35 years, were enrolled. Of the enrolled participants, 17 were in the follicular phase and 16 were in the luteal phase of menstrual cycle; 1 participant was discontinued after enrollment for a positive screening test for *Chlamydia trachomatis*. Eligible women were healthy, HIV negative, non-pregnant and reported regular menstrual cycles every 21–35 days. Women were excluded if within 30 days of enrollment they: 1) used any hormonal or intrauterine contraceptive methods; 2) underwent any surgical procedure involving the pelvis (including biopsy); 3) were diagnosed with any urogenital tract infection; 4) used any vaginal or systemic antibiotics, oral or vaginal steroids, or any vaginal product or device (including spermicide, microbicide, douche, sex toy, cervical cap, menstrual collection device,

diaphragm, or pessary) except tampons and condoms. Women were also excluded if they used depot medroxyprogesterone acetate within 10 months of enrollment, were pregnant or breastfeeding within 60 days of enrollment or had a new sex partner within 90 days of enrollment. Exclusion criteria included having unprotected heterosexual intercourse since last reported menses, having vaginal or anal intercourse within 36 hours prior to the enrollment study visit, having a prior hysterectomy or malignancy of the cervix or uterus, and having any history of immunosuppression, including immunosuppression associated with chronic disease. Medical, gynaecologic and sexual histories were obtained and screening procedures were conducted including: urine pregnancy testing; rapid HIV screening (OraQuick®, OraSure Technologies, Bethlehem, PA, USA); collection of genital tract swabs for detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* (Hologic Inc., San Diego, CA, USA) and rapid testing for *Trichomonas vaginalis* (OSOM, Sekisui Diagnostics, Lexington, MA, USA). Participants were enrolled on the same day as screening when all eligibility criteria were met including no vaginal bleeding on exam and being in the follicular (day 3–12) or luteal (≤10 days prior to anticipated start of menses) phase of their menstrual cycle by self-report. Given the low-risk study population, participants with no clinical signs of genital infection were enrolled with pending screening tests and were discontinued post-enrollment if a screening test rendered them ineligible. Final group allocation to phase of menses was based on UPLC/MS/MS serum hormone analysis with follicular phase serum progesterone <1000 pg/mL and luteal phase serum progesterone >2000 pg/mL.

Five biopsy samples from the genital tract were obtained per participant: two vaginal biopsies from the upper vagina, two cervical biopsies from the squamocolumnar junction, and one endometrial biopsy. Vaginal and cervical biopsies were obtained with a standard gynaecologic biopsy instrument and each measured approximately 2 x 3 x 2 mm. The endometrial biopsy was obtained using a standard endometrial sampler (Pipelle®, Cooper Surgical, Trunbull, CT,

USA). Participants were given the option of a cervical anesthetic injection with 10cc of 1% lidocaine solution prior to the endometrial biopsy. If this was elected, it was administered after the vaginal and cervical biopsies were obtained. One of each of the vaginal and cervical biopsies and half of the endometrial biopsy sample were each placed in 1 mL RNALater and stored at 4°C overnight before being transferred to −80°C. The second vaginal and cervical biopsies and the remaining half of the endometrial biopsy sample were separately placed into histology cassettes and incubated in 10% formalin fixative for 24 hours at room temperature. The samples were then transferred to 70% ethanol solution at room temperature. Blood samples were also evaluated by UPLC/MS/MS for quantification of estrogens and progestogens as previously described at the Magee-Womens Research Institute, Pittsburgh, PA, USA (14). Serum progesterone was evaluated by the hospital clinical laboratory. All subsequent analyses of IFNε and related parameters, including gene expression analysis and IHC quantification, were conducted by researchers in the Hudson Institute of Medical Research in Melbourne, Australia, who were blinded to cycle stage of all participants to ensure completely unbiased analysis was conducted. Once all analysis techniques and quantification were complete, researchers in the Magee-Womens Research Institute, Pittsburgh, PA, USA informed the researchers in the Hudson Institute of the cycle stage of each participant and subsequent graphing and statistical analysis was performed.

Cohort 2: For primary uterine epithelial cell cultures for ex vivo studies, human endometrial specimens were obtained from four ovulating women in proliferative stage of menstrual cycle undergoing hysterectomy or endometrial biopsy for non-endometrial benign pathologies at Monash Health following written informed consent and with approval from the Monash Health Human Research Ethics Committee. Endometrial cells were isolated on the day of sampling from donors by mincing, digestion with collagenase and DNase I and filtration, as previously

described (8). UECs were cultured for 3 days in phenol-free DMEM-F12 (Gibco, Invitrogen) supplemented with 10% v/v charcoal-stripped FCS and 1% pen/strep prior to use in experiments.

Cell culture and reagents:

We performed a series of in vitro experiments to determine the response to IFNε of various hFRT-derived cells. VK2 [vaginal (ATCC® CRL-2616™)], Ect1 [ectocervical (ATCC® CRL-2614™)] and End1 [endocervical (ATCC® CRL-2615™)] cell lines were maintained in Keratinocyte-SFM, supplemented with 0.2 ng/mL of human rEGF (epidermal growth factor), 20 μg/mL of rBPE (bovine pituitary extract) and 1% Antibiotic-Antimycotic (Gibco, Life Technologies). ECC-1 endometrial epithelial cells (ATCC) were cultured in DMEM:F12 Glutamax (Gibco, Invitrogen) supplemented with 1% pen/strep and 10% v/v FBS and validity was routinely tested using Short Tandem Repeat (STR) DNA profiling of human cell lines per ATCC guidelines. For stimulation, cells were plated at 1.5 x 10^5 cells/well in a 12 well plate and stimulated for 3h with 100 IU/mL recombinant IFNε (made as described previously (15)) or IFNβ (REBIF, Merck).

Reporter Gene Assays:

The human *IFNE* promoter (ATG −1200 bp) was cloned into the NheI/XhoI restriction site of the luciferase-reporter plasmid, promoter-less pGL3 basic vector (Promega, USA). Cells from an endometrial cancer cell line, ECC1s (ATCC® CRL-2923™), were plated in a 96-well plate (2 x 10^4/well) 24 hr prior to transfection with 60 ng of *IFNE* promoter construct using Lipofectamine 3000 reagent (ThermoFisher, Australia). TK Renilla was used to normalize for transfection efficiency and an appropriate pEF-BOS empty vector plasmid was used to

maintain a constant amount of DNA. Transfected cells were lysed using Reporter Lysis Buffer (Promega, USA) and assayed for luciferase and Renilla activity using luciferase assay reagent (Promega, USA) and Renilla substrate. Luminescence readings were detected using FLUOstar Optima (BMG Technologies) corrected for Renilla and expressed as fold induction over empty vector control values.

Gene expression analysis:

RNA was extracted using TRI reagent and the RNeasy Kit (QIAGEN, Melbourne, Australia) from biopsy samples obtained from the cross-sectional cohort described above. cDNA was synthesised following DNase treatment (Promega, USA) using MMLV reverse transcriptase and random hexamers (Promega, USA). *Biomark Fluidigm qPCR:* Gene expression was analysed using the Biomark Fluidigm system. Ct values from the Biomark qPCR were calculated using the Biomark Fluidigm Real Time PCR Analysis Software. Comparative analysis was performed using RStudio with the HTqPCR package (16), including methods for Principle Component Analysis, visualization, as well as Spearman correlation. Results were normalised to the housekeeping genes *HMBS* and *RPLPO*, which were confirmed to be stably expressed across sample types, and the average of these genes was used to normalise gene expression data. Unreliable probes were removed as defined by post normalised Ct values >10 or undetected in greater than 30% of samples. Taqman probe IDs for genes analysed are listed in Supplemental Table 1. *SyBr green qPCR:* For in vitro studies using cell lines, RNA was extracted as above and qPCR was performed using SYBR green (ABI, ThermoFisher, Australia) on an Applied Biosystems 7900HT Fast Real-Time PCR Machine. Primer sequences are listed in Supplemental Table 2. Expression was calculated using the $2^{-\Delta\Delta CT}$ method using *18S* as endogenous control and relative to control conditions.

ELISA:

Expression level of IFNε in cervicovaginal lavage (CVL) of the 32 participants were determined using a validated prototype sandwich ELISA co-developed in collaboration with PBL assay science (manuscript in preparation, S. Lim, P.Hertzog, T Lavoie and M. Skawinski) with detection range of 3.9 to 250 pg/mL. Experiments were conducted as per manufacturer's protocol with the supplied buffers and reagents. CVLs were diluted 2-fold to minimise "matrix" interference on the ELISA assay. Diluted samples were added into the ELISA with precoated anti-hIFNε capture mAb, and incubated at 25°C, 450 rpm for 1 h. The plate was washed, and biotinylated anti-hIFNε capture mAb was added and incubated at 25°C, 450 rpm for 1 h. Thereafter, the plate was washed three times before adding streptavidin-horseradish peroxidase conjugate, and incubated for a further 1 h, at 25°C, 450 rpm. The plate was washed four times, and TMB substrate was added. The plate was incubated at ambient room temperature, protected from light for 30 mins. The reaction was terminated using sulfuric acid solution, and OD 450 nm readings were obtained using BMG Labtech FLUOstar omega plate reader. The details of this ELISA have been submitted for publication in another journal as part of a larger study. (Lim, S. Hertzog, P.J. et al). Expression of IL-15 and IL-6 in CVLs from n=32 participants were determined using Luminex multi-plex assays. CVL was diluted 1:1 in assay buffer and analysed using a Human Cytokine assay panel (Merck) as per manufacturer's instructions. Supplied standards and quality controls were included. All assays were performed in duplicate with overnight incubation at 4ºC and read using a Bioplex 200 analyser (Biorad). Mean concentrations were interpolated from a 5-parameter fit standard curve.

Immunohistochemistry analysis and quantification:

Multi-core tissue arrays were created at the Monash University Histology Platform from hFRT biopsy samples from the Pittsburgh cohort; each array contained matched vaginal, ectocervical and endometrial tissue from up to nine participants included in this study in order of recruitment into the study and prior to knowledge of the stage of menstrual cycle of the participants. Thin sections (~4 µM) of each tissue array block were cut and adhered onto Superfrost™ Plus glass slides (4951PLUS4, ThermoFisher, Australia). Sections were dehydrated using a series of 100% xylene, 100% ethanol, 70% ethanol and MilliQ water solutions. Heat induced antigen retrieval was performed using a 10 mM Trizma® base (T6066, Sigma-Aldrich) 1 mM EDTA buffer at pH 9.0. Sections were blocked using CAS-block (00-8120, Invitrogen), for 1 hour at room temperature, then incubated overnight at 4ºC with the following primary antibodies: rabbit anti-human IFNε (NBP1-92018, Novus Biologicals), used at 0.5 µg/mL; mouse anti-human estrogen receptor α (IR657, Dako), provided at ready-to-use concentration; and mouse anti-human progesterone receptor (M3569, Dako), used at 1.56 µg/mL; all diluted in CAS-block. Corresponding isotype controls were rabbit IgG (I-1000, Vector Laboratories), used at 0.5 µg/mL; and mouse IgG1 (X0931, Dako), used at 1.56 µg/mL; both diluted in CAS-block. Sections were washed with 0.05% Tween-PBS for 15 minutes and incubated with 60 µg/mL biotinylated secondary antibodies (BA-1000, anti-rabbit and BA9200, anti-mouse, Vector Laboratories) for 1 hour at room temperature. Slides were washed in 0.05% Tween-PBS for 10 minutes, incubated for 45 minutes with VECTASTAIN Elite® ABC-HRP Kit, an avidin-biotinylated peroxidase H complex (PK-6100, Vector Laboratories), washed again for 10 minutes in 0.05% Tween-PBS, and DAB substrate (GV825, Dako) was applied for 30 seconds to initiate precipitate formation/colour development via peroxidase activity. Enzyme activity was stopped using distilled water. Coverslips were applied to slides with DPX mounting media (Merck) and allowed to dry overnight.

Slide scanning and image analysis:

High-resolution digital scans were acquired using the Aperio Scanscope AT Turbo (Leica Biosystems) at the Monash Histology Platform. Quantification was performed using Aperio ImageScope (v12.3.0.5056, Leica Biosystems) with the Aperio Cytoplasm Algorithm (v2, Leica Biosystems). The epithelium, glands, and stroma were delineated and assessed independently of each other for all staining. For quantification of IFNε expression by IHC, we used the positive pixel count (v9) algorithm to measure the intensity of the marker (brown signal) in epithelial and stromal areas of tissue for each participant. The positive count algorithm, which quantifies the amount of a specific stain present in a scanned slide image, was used to determine relative intensity values. As hormone receptor staining showed distinct cytoplasmic and nuclear staining patterns, an H-score for nuclear and cytoplasmic staining was obtained using the Aperio Nuclear and Cytoplasmic algorithms; each of these calculates an H-score based on the following classification of staining as follows: 0, none; 1+, weak; 2+ moderate; 3+, strong and then generation of the H-score using the formula: 1*(%1+) + 2*(%2+) + 3*(%3+); therefore a maximum H-score of 300 would indicate that 100% of cells staining as 3+. All quantification was performed by researchers who were blind to the cycle stage of the participants.

Statistics:

For Fluidigm analysis, statistically significant gene changes were determined between conditions using linear modelling, employing the limma statistical package (17). An empirical Bayes moderated t-test was used, with a 1.5-fold change cut off and Benjamini-Hochberg correction for false discovery. Gene expression was considered significant at $p<0.05$. For all

other data, statistical analyses were performed using GraphPad Prism (v9.3.0, La Jolla, CA, USA). Mann-Whitney U-tests and Kruskal-Wallis testing with Dunn's multiple comparison analysis were used as indicated. Spearman's rank correlation coefficient analysis was used for all correlation analysis.

Study Approval:

The University of Pittsburgh Institutional Review Board, USA (cohort 1) and the Monash Health Human Research Ethics Committee, Australia (cohorts 1 and 2) approved this study. Signed informed consent was collected from each participant prior to study participation.

Author Contributions

NMB led the experimental work and contributed to planning and writing of the manuscript; SLA led the planning, execution and interpretation of clinical studies performed at University of Pittsburgh/Magee-Womens Research Institute and contributed towards writing of the manuscript; SUH processed samples and performed and interpreted IHC experiments; HEC, LJG and RC contributed toward the design and analysis of gene expression arrays; IP performed in vitro type I IFN experiments; ST conducted experimental work and developed protocols under supervision of SM; NEM contributed towards experimental and conceptual aspects of the project; SM contributed to planning and interpretation of hormone data and contributed towards writing of manuscript; PJH led the intellectual development of overall project, contributed to interpretation of data and writing of the manuscript.

Acknowledgements

The authors would like to acknowledge Dr Rebecca Smith and Dr Deborah Bianco for assistance. The authors also acknowledge the scientific and technical assistance of Monash Histology Platform, Department of Anatomy and Developmental Biology, Monash University and the Monash Health Translational Precinct Medical Genomics Facility – Fluidigm Single Cell and High Throughput Centre. The authors would also like to thank Dr Tracey Edgell for guidance regarding Luminex assays. This work was supported by the Bill and Melinda Gates Foundation project OPP1108501 and an Australian National Health and Medical Research Council Fellowship (APP1117527) to Paul Hertzog.

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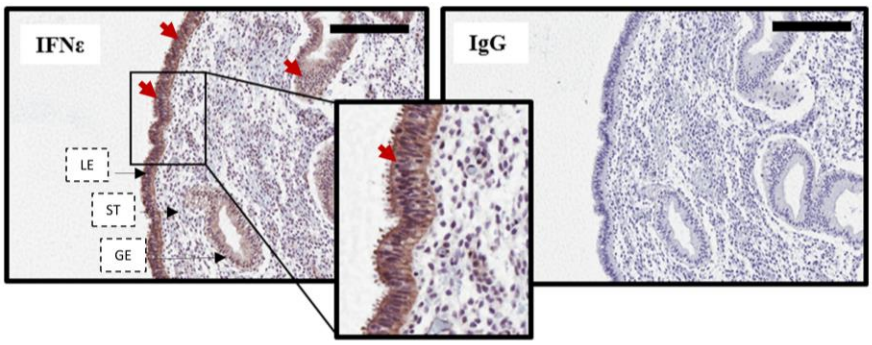
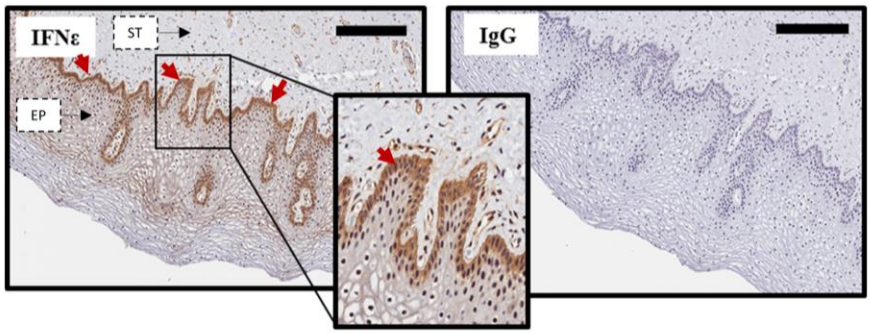
(A) Endometrium



(B) Ectocervix



(C) Vagina

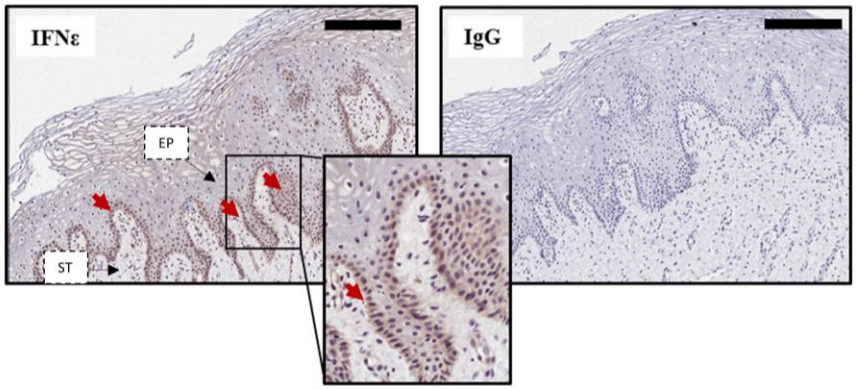


Figure 1. Distinct expression patterns of IFNε in upper and lower hFRT mucosa

Representative images of IFNε expression in sections from matched biopsy samples from n=33 women. Sections from the (A) endometrium, (B) ectocervix and (C) vagina were stained for expression of IFNε (brown staining, highlighted with red arrows) or IgG control. Bar, 200 μM. LE: luminal epithelium; GE: glandular epithelium; EP: epithelium; ST: stroma.

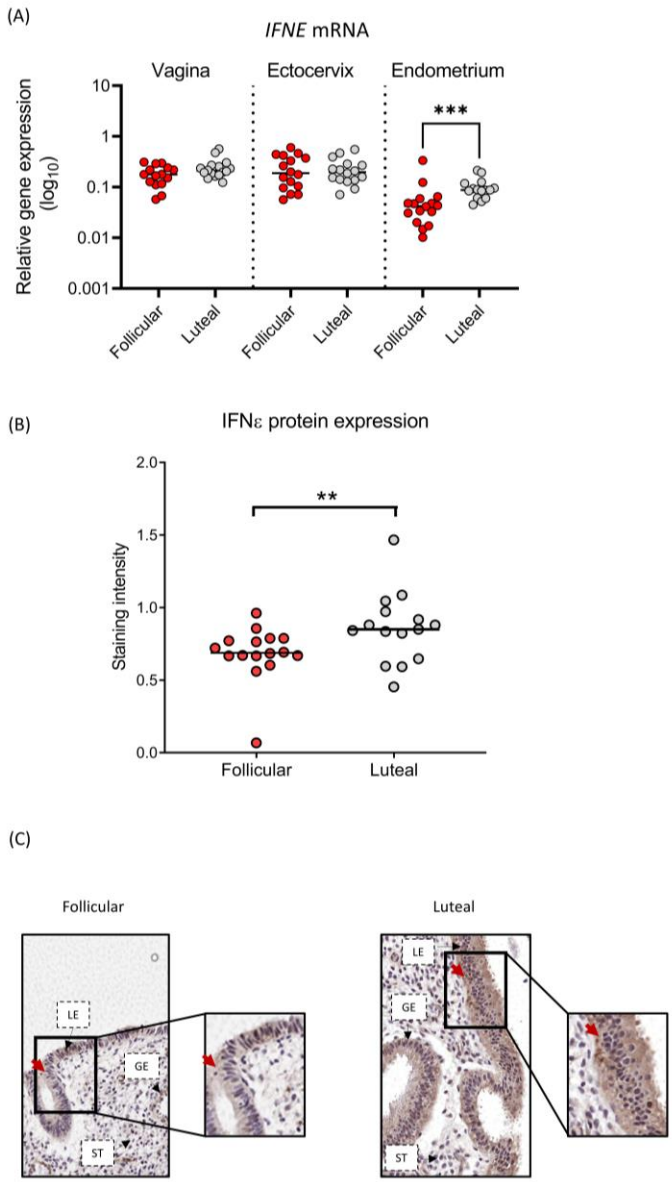
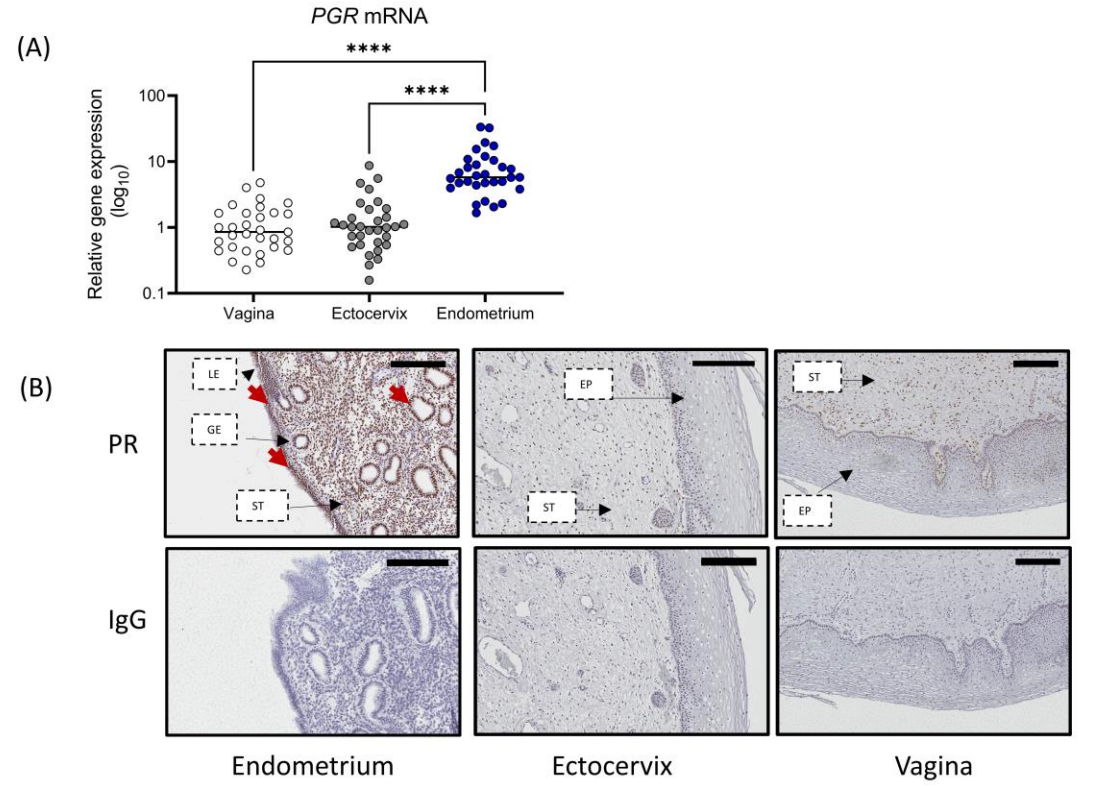
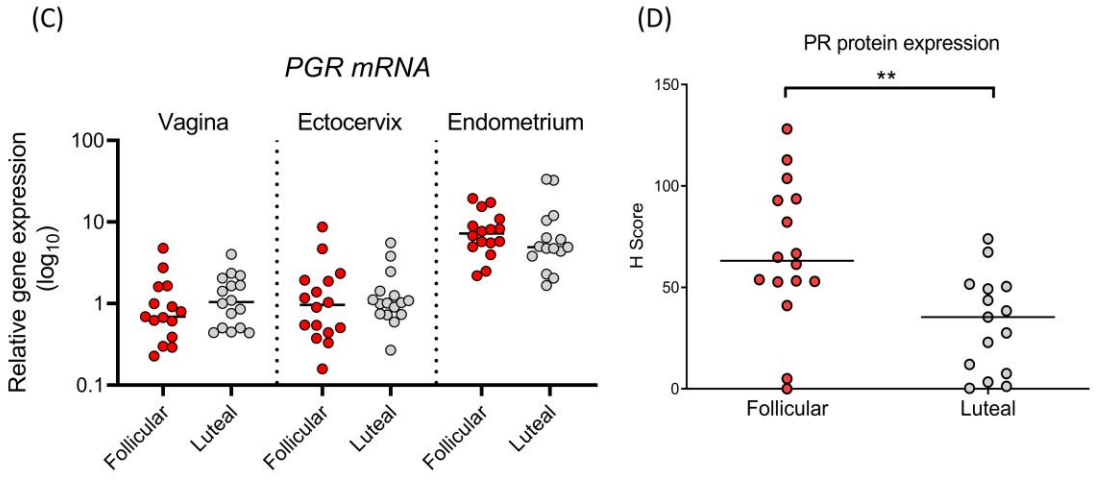


Figure 2. Cyclic variation of IFNε expression only in the upper hFRT (A) *IFNE* mRNA expression, as determined by qPCR, in vaginal, ectocervical and endometrial biopsy samples stratified into follicular (n=16) and luteal (n=16) stages of menstrual cycle. Quantification (B) and representative IHC images (C) of endometrial epithelial IFNε staining intensity in women in follicular or luteal stage of menstrual cycle using the Aperio positive pixel count algorithm to generate intensity values for staining. IFNε staining is highlighted with red arrows. Significance determined using either Kruskal-Wallis testing with Dunn's multiple comparison analysis (A) or Mann-Whitney U-test (B); *p<0.05, **p<0.01. LE: luminal epithelium; GE: glandular epithelium; ST: stroma.


(A)
PGR mRNA
****
****
Relative gene expression (log10)
100
10
1
0.1
Vagina
Ectocervix
Endometrium
(B)
PR
IgG
LE
GE
ST
EP
Endometrium
Ectocervix
Vagina


(C)
PGR mRNA
Vagina
Ectocervix
Endometrium
Relative gene expression (log10)
100
10
1
0.1
Follicular
Luteal
Follicular
Luteal
Follicular
Luteal
(D)
PR protein expression
**
H Score
150
100
50
0
Follicular
Luteal

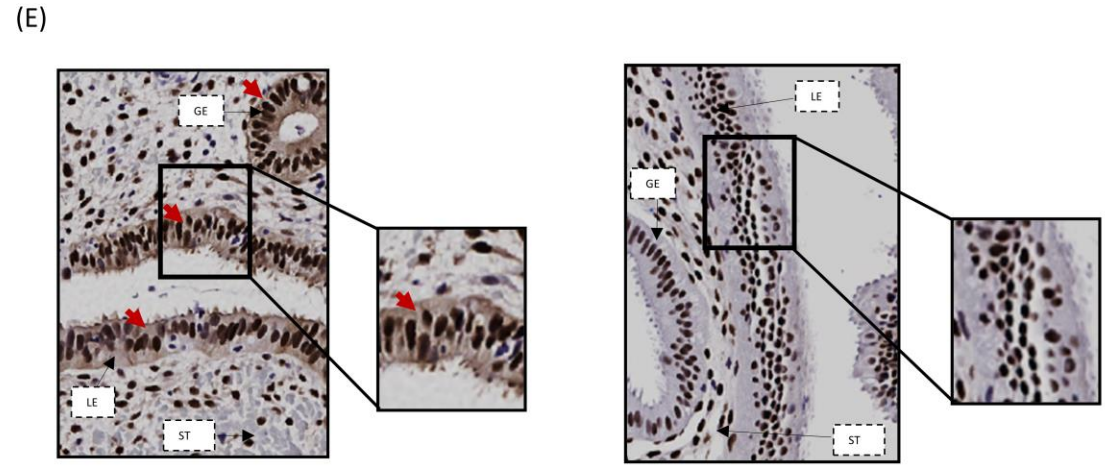

(E)
GE
LE
ST
LE
GE
ST

Figure 3. Expression of PR selective and cyclic changes only in upper hFRT (A) *PGR* mRNA expression, as determined by qPCR, in vaginal, ectocervial and endometrial biopsy samples. (B) Representative images of PR expression in sections from matched biopsy samples from n=33 women. Sections from the endometrium, ectocervix and vagina were stained for expression of PR (brown) or IgG control. Bar, 200 μM. (C) *PGR* mRNA expression, as determined by qPCR, in vaginal, ectocervial and endometrial biopsy samples stratified into follicular (n=16) and luteal (n=16) stages of menstrual cycle. (D) Quantification and representative IHC images of cytoplasmic PR staining intensity in endometrial epithelial cells from women in follicular or luteal stage of menstrual cycle. H-scores for staining were generated using the Aperio cytoplasm algorithm which classifies cytoplasmic staining intensity scoring as 0, none; 1+, weak; 2+ moderate; 3+, strong and uses this to generate an H-score using the formula: 1*(%1+) + 2*(%2+) + 3*(%3+). PR staining is highlighted with red arrows. LE: luminal epithelium; GE: glandular epithelium; EP: epithelium; ST: stroma. Significance determined using either Kruskal-Wallis testing with Dunn's multiple comparison analysis (A), (C) or Mann-Whitney U-test (D); **$p<0.01$, ****$p<0.0001$.

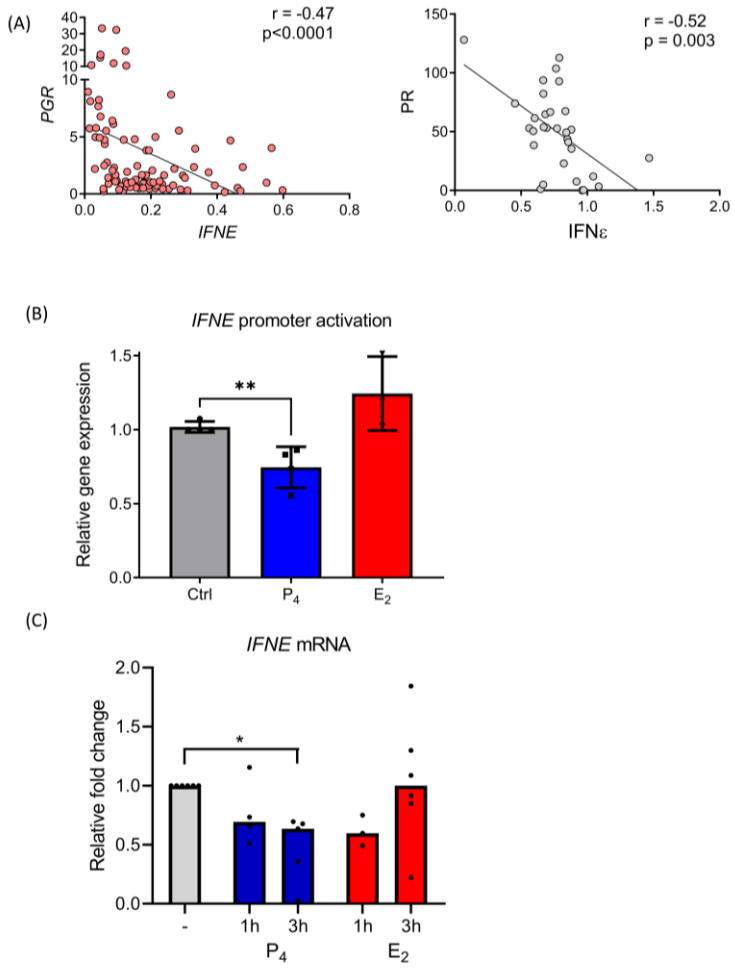


Figure 4. Regulation of IFNε by PR (A) Negative correlation of both mRNA (left) and protein expression (right) of IFNε and PR in hFRT cells. Spearman correlation analysis. (B) Luciferase reporter assay measuring activation of the human *IFNE* promoter in ECC1 cells following treatment with either 10 nM progesterone or 10 nM estrogen for 4 hours. Data from n=4 independent biological replicates, each in technical triplicate, shown as mean +SEM and analysed using Student's t-test, **p<0.01. (C) Primary uterine epithelial cells were isolated from endometrial biopsies (from up n=6 donors) and cultured for 3 days prior to stimulation for either 1 or 3 hours with 10 nM progesterone or 10 nM estrogen. *IFNE* expression was quantified using qPCR, expressed relative to expression of *18S* and fold change relative to unstimulated control. Significance determined using Kruskal-Wallis testing with Dunn's multiple comparison analysis, *p<0.05.

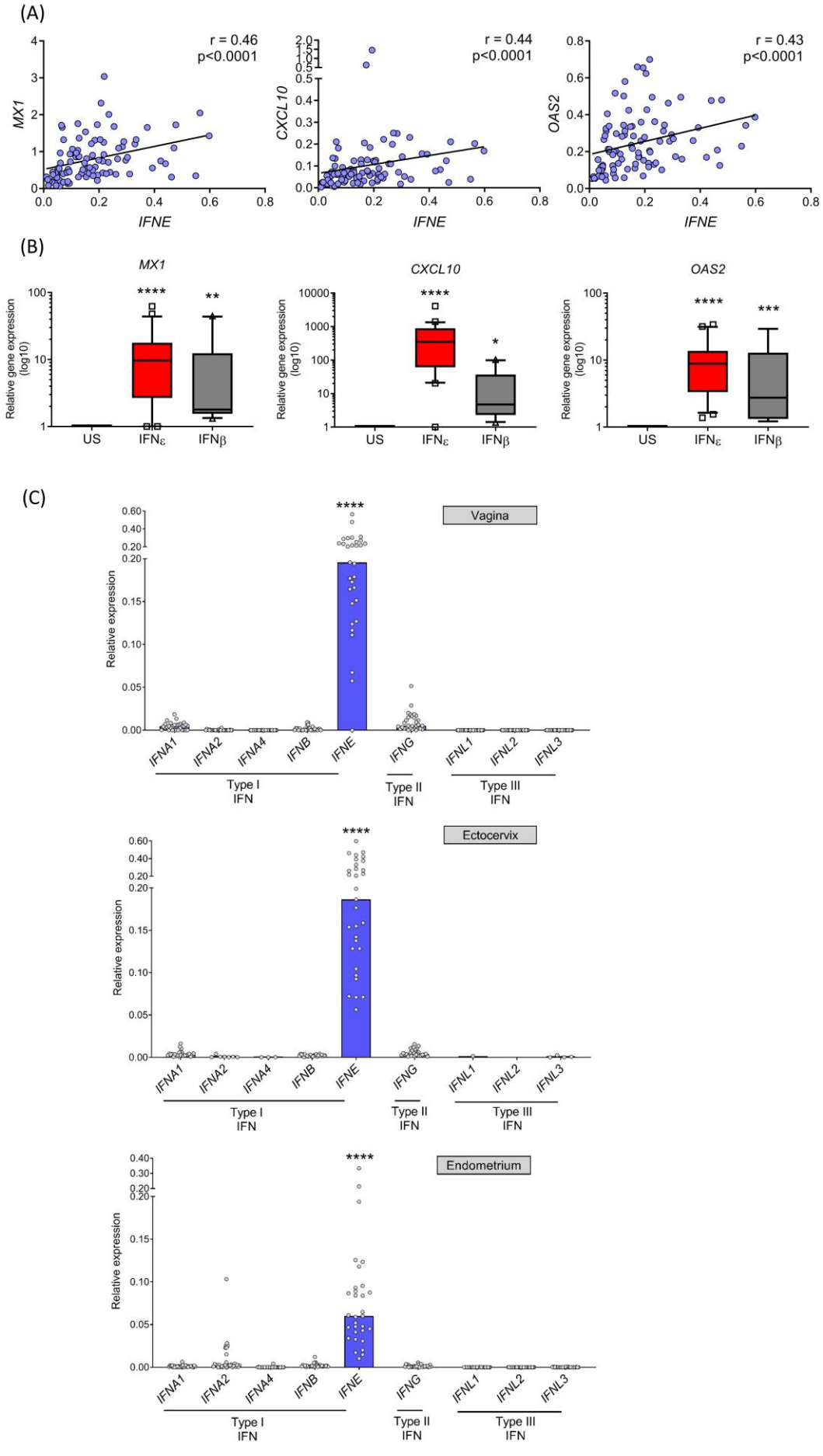

(A)
r = 0.46
p<0.0001
MX1
IFNE
r = 0.44
p<0.0001
CXCL10
IFNE
r = 0.43
p<0.0001
OAS2
IFNE
(B)
MX1
CXCL10
OAS2
Relative gene expression (log10)
US
IFNε
IFNβ
(C)
Vagina
Ectocervix
Endometrium
Relative expression
IFNA1
IFNA2
IFNA4
IFNB
IFNE
IFNG
IFNL1
IFNL2
IFNL3
Type I IFN
Type II IFN
Type III IFN

Figure 5. Exclusive expression of IFNε in hFRT regulates immune-protective IRGs (A) Spearman correlation analysis of the expression of *IFNE* with the IRGs *MX1*, *CXCL10* and *OAS2* across hFRT biopsy samples. (B) Primary uterine epithelial cells were isolated from endometrial biopsies and cultured for 3 days prior to stimulation with IFNβ (n=9) or IFNε (n=20). (C) Expression of type I IFN (*IFNA1*, *IFNA2*, *IFNA4*, *IFNB*, *IFNE*), type II IFN (*IFNG*) and type III IFN (*IL28A*, *IL28B*, *IL29*) was quantified by qPCR in matched vaginal, ectocervical and endometrial biopsy samples from n=32 women regardless of phase of menses. In vaginal samples, *IFNA1* was not detectable (N/D) in n=10, *IFNA2* N/D in n=24, *IFNA4* N/D in n=31, *IFNB* N/D in n=13, *IFNG* N/D in n=2, *IFNL1*, *IFNL2* N/D in n=31 and *IFNL3* was N/D in n=30. In ectocervical samples, *IFNA1* was N/D in n=6, *IFNA2* N/D in n=24, *IFNA4* N/D in n=28, *IFNB* N/D in n=7, *IFNG* N/D in n=1, *IFNL1* N/D in n=30, *IFNL2* N/D in n=31 and *IFNL3* was N/D in n=27. In endometrial samples, *IFNA1* was N/D in n=10, *IFNA2* N/D in n=24, *IFNA4* N/D in n=31, *IFNB* N/D in n=13, *IFNG* N/D in n=2, *IFNL1*, *IFNL2* N/D in n=31 and *IFNL3* was N/D in n=30. Gene expression was quantified using qPCR, normalised to *18S* expression and expressed relative to untreated control cells. Data analysed using Kruskal-Wallis testing with Dunn's multiple comparison analysis, ****p<0.0001. US: unstimulated; Mann-Whitney U-Test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

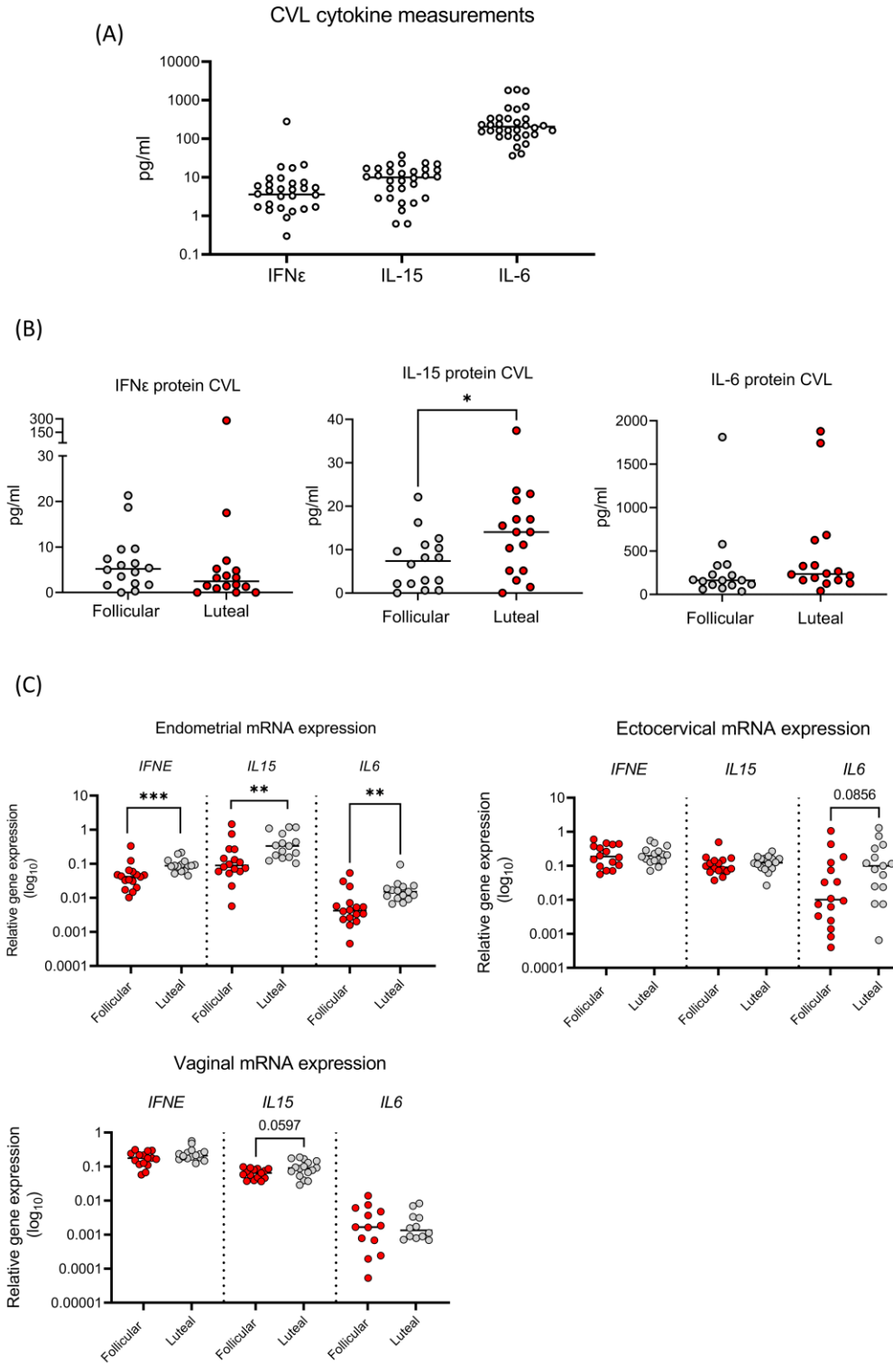

(A)
CVL cytokine measurements
pg/ml
10000
1000
100
10
1
0.1
IFNε
IL-15
IL-6
(B)
IFNε protein CVL
IL-15 protein CVL
IL-6 protein CVL
pg/ml
Follicular
Luteal
*
(C)
Endometrial mRNA expression
Ectocervical mRNA expression
Vaginal mRNA expression
IFNE
IL15
IL6
Relative gene expression (log10)
***
**
**
0.0856
0.0597
Follicular
Luteal

Figure 6. IFNε protein is expressed in cervicovaginal lavage (CVL). (A) Concentrations of IFNε, IL-15 and IL-6 were quantified in CVL fluid (n=32) using either laboratory-developed (IFNε) or commercially available (IL-15, IL-6) immunoassays. There was undetectable cytokine expression in n=4 samples for IFNε and n=2 samples for IL-15. (B) CVL IFNε, IL-15 and IL-6 expression was stratified by cycle stage into n=16 follicular and n=16 luteal. There was undetectable cytokine expression for IFNε in n=1 follicular and n=3 luteal and IL-15 n=1 follicular and n=1 luteal. (C) *IFNE, IL15* and *IL6* mRNA expression, as determined by qPCR, in endometrial, ectocervical and vaginal biopsy samples stratified into follicular (n=16) and luteal (n=16) stages of menstrual cycle. Mann-Whitney U-Tests were applied to determine cyclic differences for each gene or protein of interest, *$p<0.05$, **$p<0.01$,***$p<0.001$.

Table 1. Demographic characteristics for participants in Pittsburgh cohort

	Follicular N=17	Luteal N=16	p-value[A]
Age, years (mean, SD)	24.6 (4.6)	25.1 (3.6)	0.78[B]
Ethnicity			>0.99
Hispanic	1 (5.9%)	0	
Non-Hispanic	16 (94.1%)	16 (100%)	
Race			0.54
White	13 (76.5%)	9 (56.3%)	
Black	3 (17.6%)	6 (37.5%)	
Bi- or multi-racial	1 (5.9%)	1 (6.3%)	
Marital status			0.23
Single, never married	17 (100%)	14 (87.5%)	
Married	0	1 (6.3%)	
Divorced	0	1 (6.3%)	
Partner status			0.20
No current partner	5 (29.4%)	7 (43.8%)	
Does not live with partner	5 (29.4%)	7 (43.8%)	
Lives with partner	7 (41.2%)	2 (12.5%)	
Highest school level completed			0.29
HS graduate or GED	7 (41.2%)	3 (18.8%)	
College graduate	7 (41.2%)	11 (68.8%)	
Graduate school graduate	3 (17.6%)	2 (12.5%)	
Tobacco smoker	4 (23.5%)	0	0.10
Marijuana smoker	3 (17.6%)	0	0.23
Gravidity			>0.99
0	13 (76.5%)	13 (81.2%)	

1 or more	4 (23.5%)	3 (18.8%)	
Parity			>0.99
0	14 (82.4%)	13 (81.2%)	
1 or more	3 (17.6%)	3 (18.8%)	
Condom use			>0.99
Never	5 (29.4%)	5 (31.3%)	
Sometimes	4 (23.5%)	3 (18.8%)	
Always	8 (47.1%)	8 (50.0%)	
Typical number of sexual acts per month			0.34
None	6 (35.3%)	7 (43.8%)	
1–4	10 (58.8%)	6 (37.5%)	
5 or more	1 (5.9%)	3 (18.8%)	
Progesterone, pg/mL (median, IQR)	49.0 (39.5, 318.5)	6171.0 (2998.0, 9774.0)	<0.001[C]
Estradiol, pg/mL (median, IQR)	82.0 (36.0, 167.0)	126.0 (96.5, 173.2)	0.06 [C]
Serum Progesterone, nmol/L (median, IQR)	1.2 (0.8, 2.6)	21.7 (14.5, 40.1)	<0.001[C]

[A] Fisher's exact test unless otherwise noted

[B] Student's t-test

[C] Mann-Whitney U-test