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Endometrial stromal fibroblasts from women with polycystic ovary syndrome have impaired progesterone-mediated decidualization, aberrant cytokine profiles and promote enhanced immune cell migration in vitro

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STUDY QUESTION: Do endometrial stromal fibroblasts (eSF) in women with polycystic ovary syndrome (PCOS) (eSFPCOS) exhibit altered estrogen and/or progesterone (P4) responses, which may explain some of the adverse reproductive outcomes and endometrial pathologies in these women?

SUMMARY ANSWER: In vitro, eSF from women with PCOS exhibit an aberrant decidualization response and concomitant changes in pro-inflammatory cytokine, chemokine and matrix metalloproteinase (MMP) release and immune cell chemoattraction. In vivo these aberrations may result in suboptimal implantation and predisposition to endometrial cancer.

WHAT IS KNOWN ALREADY: The endometrium in women with PCOS has several abnormalities including progesterone (P4) resistance at the gene expression level, likely contributing to subfertility, pregnancy complications and increased endometrial cancer risk in PCOS women.

STUDY DESIGN, SIZE, DURATION: Prospective, university-based, case–control, in vitro study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cultures of eSFPCOS (n = 12, Rotterdam and NIH criteria) and eSFControl (Ctrl) (n = 6, regular cycle length, no signs of hyperandrogenism) were treated with vehicle, estradiol (E2; 10 nM) or E2P4 (10 nM/1 μM) for 14 days. Progesterone receptor (PGR) mRNA was assessed with quantitative real-time PCR (qRT–PCR) and eSF decidualization was confirmed by insulin-like growth factor-binding protein-1 (IGFBP-1) transcript and protein expression. Fractalkine (CX3CL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 6, 8 and 11, macrophage chemoattractant protein (MCP) 1 and 3, CCL5 (RANTES) and MMPs (MMP1, 2, 3, 7, 9, 10 and 12) were measured in conditioned media by Luminex multiplex assays, and chemotactic activity of the conditioned media was tested in a migration assay using CD14+ monocyte and CD4+ T-cell migration assay. Effects of IL-6 (0.02, 0.2, 2 or 20 ng/ml) or IL-8 (0.04, 0.4, 4, or 40 ng/ml) or combination (0.2 ng/ml IL-6 and 4.0 ng/ml IL-8) on 14-d decidualization were also tested. ANOVA with pre-planned contrasts was used for statistical analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: Hormonal challenge with E2P4 to induce decidualization revealed two distinct subsets of eSFPCOS. Eight eSFPCOS (dPCOS) and all eSFControl (dCtrl) cultures showed a normal decidualization response to E2P4 as determined by morphology and IGFBP-1 secretion. However, 4 eSFPCOS cultures showed blunted decidualization (ndPCOS) in morphological assessment and low IGFBP-1 levels even though all three groups exhibited normal estrogen-mediated increase in PGR expression. Interestingly dPCOS had decreased IL-6 and...
Introduction

In humans, decidualization is a progesterone (P₄) driven differentiation process essential to prepare the endometrium for successful embryo implantation and maintenance of pregnancy (Cha et al., 2012). Decidualization occurs in the secretory phase when endometrium undergoes vast and complex cellular differentiation dependent on progesterone receptor (PGR) up-regulation by estradiol (E₂), activation of P₄ signaling and convergence of cyclic adenosine monophosphate (cAMP) signaling (Aghajanova et al., 2009; Zhu et al., 2014). Glandular secretory transformation occurs in response to P₄, and perivascular endometrial stromal fibroblasts (eSF) initiate decidualization throughout the endometrium that continues to progress if conception occurs (Cha et al., 2012; Zhu et al., 2014). Decidualized eSFs undergo morphological and functional changes with glycogen and lipid accumulation in the cytoplasm, providing a source of nutrition for the developing embryo prior to placental development (Cha et al., 2012; Zhu et al., 2014). In response to P₄-mediated activation of transcription factors forkhead box protein O1 (FOXO1) and homeobox A10 (HOXA10), decidualized eSFs produce insulin-like growth factor-binding protein 1 (IGFBP-1), commonly used as a marker of decidualization. IGFBP-1 restricts trophoblast invasion and endometrial growth, in part, by inhibiting insulin growth factor 1 (IGF-1) action (Irwin and Giudice, 1998).

Several growth factors, cytokines and matrix metalloproteinases (MMPs) play a role in the decidualization process to optimize the endometrial environment for implantation where balanced trophoblast invasion, outgrowth and vasculature establishment are achieved (Staun-Ram and Shalev, 2005). Several cytokines, e.g. IL-11, LIF, IL-6 and transient immune cells, are present during decidualization and participate in the communication between differentiated endometrium and the embryo (Kojima et al., 1994; Cork et al., 2001). In addition to cytokines, MMPs, a family of zinc-dependent proteases, are also highly expressed in decidua as they proteolyze the extracellular matrix, allowing tissue growth and remodeling in response to normal proliferative and differentiative signals (Godbole et al., 2011). MMPs allow for histoarchitectural modifications necessary for embryo attachment and invasion where suppression of endometrial MMPs is necessary to maintain endometrial stability preventing excessive invasion of extravillous trophoblasts and abnormal placental development (Visse and Nagase, 2003; Licht et al., 2007; Godbole et al., 2011). In endometrium, P₄ normally suppresses MMP expression directly, up-regulates tissue inhibitors of metalloproteinases (TIMPs) and modulates cytokine activity (Higuchi et al., 1995; Bischof et al., 1998; Wissink et al., 1998)—all of which control MMP actions, although in P₄-resistant disorders, this suppression of MMPs is not observed.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive aged women, affecting around 10% of this population and characterized as menstrual dysfunction, anovulatory infertility, hyperandrogenism and insulin resistance (Amsterdam ESHRE/ASRM-Sponsored 3rd PCOS Consensus Workshop Group 2012). In PCOS impaired follicle maturation and consequent anovulation cause a chronic P₄-deficient state that affects the endometrial milieu. Furthermore, even with ovulation (and thus P₄ acting on endometrium), the PCOS endometrium has several abnormalities including altered steroid receptor and glucose transporter 4 (GLUT4) expression as well as P₄-resistance (Apparao et al., 2002; Villavicencio et al., 2006; Savarirayan et al., 2011; Carvajal et al., 2013). Moreover, we as well as others have recently reported an inflammatory milieu in PCOS endometrium with an increased inflammatory profile in the proliferative phase and decreased uterine natural killer cell influx in late secretory phase. This is likely to contribute to infertility, increased prevalence of pregnancy complications and endometrial cancer in these women (Matteo et al., 2010; Haoula et al., 2012; Piltonen et al., 2013; Barry et al., 2014; Naver et al., 2014).

Since P₄-regulation of endometrium during the secretory phase involves a complex network of differentiative cues with growth and pro-inflammatory factors and MMPs, we hypothesized that eSF from women with PCOS may show aberrant P₄-responsiveness during in vitro decidualization and concomitant altered production of pro-inflammatory cytokines and MMPs.
Materials and Methods

Study subjects and tissues

Endometrial tissue biopsies were obtained through the National Institute of Health (NIH)/University of California, San Francisco (UCSF), Human Endometrial Tissue and DNA Bank in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from all participants in the UCSF Center for Reproductive Health, and the study was approved by the UCSF Committee on Human Research. For the cytokine challenge, tissue samples were acquired from the Department of Obstetrics and Gynecology, University of Oulu, Finland. The tissue collection was approved by the hospital ethics committee and informed consent was obtained from all participants. Endometrial biopsies (Pipelle, Cooper Surgical, Shelton, CT) were collected, and eSF were isolated from 12 women with PCOS fulfilling both Rotterdam and NIH criteria and 11 control women (Table I). All PCOS subjects had normal 17-hydroxyprogesterone, prolactin and thyroid hormone levels. Control samples were obtained from healthy volunteers and women undergoing benign gynecological surgery. All controls reported menstrual cycles with regular interval (25–35 days) and no indication of having PCOS. Only one control subject was a smoker. None of the subjects was exposed to hormonal medications for at least 3 months prior to tissue sampling and were confirmed not pregnant during the time of study participation. The clinical summary of the study participants and tissue samples is shown in Table I.

Primary eSF cultures and decidualization experiments

eSF Isolation and culture

Endometrial samples were digested with collagenase (6.4 mg/ml collagenase type I and 100 U/ml hyaluronidase in Hank’s Buffered Salt Solution with Ca²⁺ and Mg²⁺) following filtration using an optimized protocol as previously described (Aghajanova et al., 2009; Chen et al., 2013). Stromal cell cultures we established as reported earlier with purity validated by vimentin and e-cadherin immunohistochemistry (Chen et al., 2013). Cells were cultured in growth medium (phenol red-free medium of 3:1 high-glucose Dulbecco’s Modified Eagle’s Medium [DMEM]/MCDB-105 (a fibroblast-lineage supplement medium containing trace elements and amino acids), 0.676 mM sodium pyruvate, 10% (v/v) charcoal-stripped fetal bovine serum (FBS), 1% (w/v) penicillin-streptomycin mix, 50 μg/ml gentamycin, 5 μg/ml insulin) which was renewed approximately every 2–3 days for up to the third passage and thereafter the cells were used in experiments.

Decidualization

One hundred thousand eSFs were plated into 12-well plates in duplicate and cultured in growth medium. When confluent, cells were incubated for 24 h in low-serum medium (3:1 high-glucose DMEM/MCDB-105, 0.75 mM sodium pyruvate, 50 μg/ml gentamycin, 2% FBS) prior to hormone treatment. For decidualization, cultures of eSFPCOS (n = 12) and eSFCtrl (n = 6) were treated with 0.1% ethanol vehicle (veh), estradiol (E2, 10 nM) or E2P4 (10 nM/1 μM) in low-serum medium for 14 days, with feeding every other day as previously described (Aghajanova et al., 2009). At day 14 conditioned media were collected and stored at −80°C, and cells were trypanized, counted (TC20 Automated Cell Counter, Bio-Rad), to adjust the cell counts for cytokine and MMP measurements) and stored at −80°C for RNA isolation.

Morphological assessment

Cell morphology was assessed at every media change using inverted phase contrast light microscopy. At day 14, prior to cell harvesting all wells were imaged (Zeiss Thornwood, NY) and the morphological changes of eSF were estimated by two different observers. Decidualized eSF cultures were classified based on cell shape as decidualized (spindle-like) or non-decidualized (polygonal/cobblestone).

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction

Total RNA was isolated from cultured eSF (Nucleospin RNA purification kit, Machery Nagel, Bethlehem, PA) following the manufacturer’s protocol, including DNase treatment. Purity of all RNA samples was confirmed (NanoDrop, Wilmington, DE) and cDNA were synthesized utilizing the Taqman Preamp Master mix (Life Technologies, Grand Island, NY), utilizing a 14-cycle enrichment protocol, as previously described (Piltonen et al., 2013).

To determine the hormonally-regulated gene expression in eSF in different study groups, PGR (a measure of estrogen responsiveness) and IGFBP-1 (a measure of progesterone responsiveness) mRNA expression was measured using 20 ng cDNA, and 1 μM for each primer pair. Amplification was performed using the Stratagene MX3005p (Agilent, Santa Clara, CA) Thermo-cycler with variables previously described (Aghajanova et al., 2009). Dissociation curves for both target and housekeeping genes were utilized to ensure the absence of primer dimers and other non-specific amplification. Primers were designed by Fluidigm and optimized for quantitative real-time polymerase chain reaction (qRT–PCR) following the Fluidigm Biomark guidelines on mRNA amplification, including primer amplification efficiency, amplicon size and appropriate dissociation temperatures governing mRNA amplification. The amplification conditions were compliant with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Johnson et al., 2014). All target gene expressions were normalized to YWHAZ, a highly conserved gene in PCOS endometrium (Sadek et al., 2012), coding the 14-3-3 signal transduction protein. No significant changes in YWHAZ expression were observed across dCtrl, dPCOS, ndPCOS, suggesting high stability in expression across treatment groups. The comparative (delta-delta) Ct method was used to measure relative gene expression (ABI User bulletin 2).

Protein measurements

ELISA

IGFBP-1 in conditioned media was measured using ELISA (Alpha Diagnostics, San Antonio, TX). Samples were assayed in duplicate and both standard curve and pre-measured IGFBP-1 recombinant protein controls were run in each experiment. Levels of IGFBP-1 for each sample were normalized to total cell count for each sample.

Luminex multiplex assays

A custom multiplex Luminex kit (EMD Millipore, Billerica, MA) was utilized to assay the quantity of cytokines and MMPs secreted into the conditioned media. Targeted cytokines included fractalkine (CX3CL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL), 8, 11, monocyte chemoattractant protein 1 (MCP-1; CCL2) and 3 (CCL7), and Regulated upon Activation, Normal T-cell Expressed, and Secreted Chemo-kine (C-c motif) ligand 5 (CCL5; RANTES). In addition, MMP 1, 2, 3, 7, 9, 10 and 12 were also measured. All protocols were based on the manufacturer’s specifications and performed as previously described (Chen et al., 2013). All experiments were conducted in duplicate. To determine inter-assay variability, several samples were run on two or more kits and showed an inter-assay variability <5%.

Migration assays

Immune cells utilized for migration assays were isolated fromuffy coats purchased from the Stanford Blood Bank (Palo Alto, CA). Buffy coats were
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PE, proliferative phase; S, secretory phase; HA, biochemical hyperandrogenism; OA, oligo-amenorrhea; A, amenorrhea; PCO, polycystic ovaries; IGT, impaired glucose tolerance in OGTT; N/A, not acquired.
aSamples used in the primary decidualization study.
bSamples used in cytokine challenge test.
CD14

CD14

CD14

CD14

impairment during decidualization

Cytokine challenge during decidualization

Statistics

Statistics

Results

Clinical characteristics

Impaired decidualization in eSFPCOS

Morphology

qRT–PCR analysis of hormone-responsive genes

IGFBP-1

Decidualization was also assessed by measuring secreted IGFBP-1 protein (ELISA) into conditioned media. For vehicle and E2 treated cells no IGFBP-1 was detected, as expected (data not shown). In contrast, all six control samples treated with E2P4 showed high IGFBP-1 concentration (mean ± SD; 118.94 ± 4.12 ng/10⁵ cells) validating the IGFBP-1 mRNA was also measured after vehicle or E2P4 treatment. The data showed that the IGFBP-1 mRNA expression in response to E2P4 in ndPCOS was significantly decreased (P < 0.05) compared with both dCtrl and dPCOS (Fig. 1B).
Altered cytokine secretion in eSFPCOS

IL-6 and 8, IL-11, RANTES, MCP-1 and 3, GM-CSF and Fractalkine were measured in the conditioned media (Supplementary data, Table SI).

IL-6 and 8

The basal IL-6 secretion decreased in response to E2 and/or E2P4 administration (Fig. 2A and Supplementary data, Table SI) within dCtrl (E2P4, P = 0.03) and ndPCOS groups (E2 and E2P4, P < 0.001), but not in dPCOS. Interestingly, basal IL-6 secretion was lower in dPCOS compared with dCtrl (P = 0.03); whereas, basal IL-6 secretion was increased in ndPCOS versus dCtrl (P < 0.0001). Furthermore the steroid hormone response of IL-6 was blunted in ndPCOS as E2 and E2P4 treated cells presented with increased IL-6 secretion compared with dCtrl and dPCOS (E2 and E2P4, P < 0.01) (Fig. 2A, Supplementary data, Table SI). Basal IL-8 levels were comparable between the study groups; however, the E2P4 treated IL-8 levels were higher in ndPCOS than in dCtrl and dPCOS (P < 0.05, P < 0.01, Fig. 2B).

MCP-1, RANTES and GM-CSF

Basal MCP-1 secretion was increased in ndPCOS versus dPCOS (P < 0.01, Fig. 2C, Supplementary data, Table SI) and a similar trend was observed compared with dCtrl (P = 0.06). Treatment with E2 and E2P4 decreased MCP-1 secretion in ndPCOS (P < 0.05, P < 0.01). A similar trend was also observed in dCtrl and dPCOS (Fig. 2C). Similar to IL-6, basal ndPCOS RANTES secretion was increased versus dCtrl and dPCOS (both P < 0.0001, Supplementary data, Table SI). In dPCOS and ndPCOS RANTES secretion was induced by P4 compared
with basal and/or E2 treatment (P < 0.05), and a similar trend was also observed in the dCtrl group although the increase did not reach statistical significance (Fig. 2D). Moreover, the E2P4-treated ndPCOS samples had increased RANTES levels compared with dCtrl (P < 0.01) (Fig. 2D). Interestingly basal GM-CSF levels were decreased in dPCOS compared with dCtrl (Fig. 2E). E2 treatment decreased basal GM-CSF levels in dCtrl (P < 0.01), but not in the PCOS groups. E2P4 treatment decreased GM-CSF levels in dCtrl compared with basal levels; however, this decrease was not observed in dPCOS and there was only a trend in ndPCOS. On the other hand the E2P4 treatment resulted in GM-CSF levels that were statistically comparable among the groups (Fig 2E).

No statistically significant differences were observed in IL-11, MCP-3 and fractalkine levels among the study groups (Supplementary data, Table SI).

**Impaired decidualization results in high secreted MMPs by ndPCOS**

Matrix metalloproteinase 1, 2, 3, 7, 9, 10 and 12 were measured in the condition media (Supplementary data, Table SII).

**MMP2 and 3**

Basal MMP2 levels were higher in ndPCOS compared with dCtrl and dPCOS (Fig. 3A). Interestingly, regulation of MMP2 by E2 differed in all three groups: E2 treatment did not alter, increased and decreased MMP2 levels in dCtrl, dPCOS, and ndPCOS, respectively (Fig. 3A). Overall, in E2-treated cells, MMP2 levels were increased in PCOS samples compared with dCtrl. E2P4 treatment increased MMP2 levels in all groups, and the levels were higher in the ndPCOS group versus dCtrl (P < 0.001, Fig. 3A). MMP3 levels did not differ between dCtrl and dPCOS. However, basal MMP3 levels were decreased by E2P4 treatment only in the dCtrl group; whereas, levels were increased in all treatment groups in ndPCOS versus dCtrl (Fig 3B)

No statistically significant differences were found in secreted levels of MMP1, 7, 10 and 12 between the study or treatment groups. MMP9 was not detected.

**Impaired decidualization promotes immune cell migration in PCOS**

To test whether increased cytokines secreted by ndPCOS affected the migration potential of different immune cells, CD14+ monocytes and CD4+ T-cells were exposed to pooled dCtrl, dPCOS or ndPCOS condition media (Fig. 4). Both cell types showed increased migratory potential in a presence of ndPCOS media compared with the media of dCtrl or dPCOS, consistent with high chemotactic cytokine concentrations (IL-8, MCP-1 and RANTES) in ndPCOS.

**IL-6 or IL-8 did not inhibit decidualization**

As IL-6 and IL-8 levels were increased in ndPCOS samples during decidualization compared with dCtrl and dPCOS, we investigated whether IL-6 and/or 8 could inhibit decidualization. All control stromal cell samples showed typical decidualization morphology after 14-d E2P4 + cytokine challenge test regardless of concentration or cytokine(s) used. IGFBP-1 in condition media was assessed, and all samples had comparable IGFBP-1 concentrations to decidualizing control eSF (data not shown).

**Discussion**

To our knowledge, this is the first in vitro study showing impaired endometrial differentiation in response to steroid hormones, in women with PCOS. These novel data report decidualization failure in a subset of eSFs obtained from PCOS endometrium with concomitant altered cytokine and MMP profiles. We postulate that these abnormalities may contribute to endometrial pathologies common in PCOS women, i.e. subfertility, pre-eclampsia and endometrial cancer.

**Steroid hormone response of eSFPCOS**

The present data revealed a subpopulation of women with PCOS whose eSFs exhibited a blunted decidualization response to E2P4 treatment. Interestingly, the non-decidualizing phenotype was not related to any specific clinical characteristics of the patients. Although, all ndPCOS samples were from the proliferative phase and thus may reflect their hormonal (P4) response profile. The altered E2P4 response was also unrelated to PGR expression, as all study groups (dCtrl, dPCOS and ndPCOS) exhibited a robust increase in PGR gene expression in response to E2 even though expression of decidualization marker IGFBP-1 was compromised in PCOS samples. Thus, it is possible that blunted P4 response in eSFPCOS is rooted elsewhere. DNA methylation or microRNAs have been implicated in the governing of hormonal-responsive genes, including P4 mediated pathways (Lam et al., 2012; Houshdaran et al., 2014), and in this context warrant further investigation.

Decidualization and concomitant endometrial IGFBP-1 production from differentiating stromal cells are essential for embryo attachment, and thus decreased IGFBP-1 may compromise normal implantation as it regulates IGF II bioavailability at the feto-maternal interface and restricts trophoblast invasion (Giudice et al., 1993; Irwin et al., 1993; Irwin et al., 1999). In this study, the IGFBP-1 levels in dPCOS tended to be lower than in dCtrl, although this did not reach statistical significance. On the other hand, ndPCOS with failed decidualization, demonstrated morphologically as well as by low IGFBP-1 production, may explain implantation abnormalities in some women with PCOS (Gratton et al., 2002; Palomba et al., 2012). In addition to its role in embryo implantation, IGFBP-1 may play a crucial role in the pathogenesis of endometrial hyperplasia/cancer, known risks in women with PCOS, by inhibiting IGF-I and E2-stimulated epithelial proliferation (Rutanen et al., 1988; Rajkumar et al., 1996).

**Cytokines and chemokines secreted by eSFPCOS**

Cytokines and chemokines are secreted by endometrial epithelial cells and stromal fibroblasts and by stationary and transient immune cells. They participate in the dialogue between maternal and embryonic tissues and also between epithelial and stromal compartments (Cha et al., 2012; Chen et al., 2013). During the window of implantation (WOI) and the decidualization process, cytokines including the glycoprotein 130 (gp130) family members IL-11, LIF, IL-6 facilitate implantation; whereas, the chemokines IL-8, MCP-1 and RANTES recruit leucocyte cohorts to the implantation site (Dimitriadis et al., 2005). Disturbances in cytokine and chemokine expression result in absolute or partial implantation failure and abnormal placental formation in mice and humans (Cha et al., 2012; Banerjee et al., 2013).
IL-6
IL-6 is a multifunctional cytokine with a wide range of biological activities. In addition to epithelium IL-6 is expressed in endometrial stromal cells and in decidua and has an important role coordinating placental morphogenesis and trophoblast invasion (Lockwood et al., 2008; Piltonen et al., 2013). IL-6-deficient mice have reduced fertility and fewer implantation sites and in vitro IL-6 decreases mice embryo attachment and growth (Jovanovic and Vicovac, 2009; Prins et al., 2012). Women experiencing recurrent miscarriage have decreased endometrial IL-6 levels, and IL-6 expression is elevated in decidua in women with pre-eclampsia (Demir et al., 2009; Prins et al., 2012). In this study eSF IL-6 secretion was decreased in response to E2 and E2P4 in dCtrl and ndPCOS groups, demonstrating that eSF that fail to decidualize can respond in other ways to steroid hormones. Interestingly, in dPCOS basal IL-6 levels were decreased compared with controls, and they did not decrease in response to steroid hormones, similar to other groups, which may indicate

Figure 2 Multiplex protein analysis (Luminex) of different cytokines (pg/10^5 cells) after 14-days treatment with vehicle (V) or 10 nM estradiol (E2), or 10 nM E2 plus 1 μM progesterone (P4). (A) Interleukin 6 (IL-6). (B) Interleukin 8 (IL-8). (C) Monocyte chemoattractant protein-1 (MCP-1). (D) Regulated on Activation, Normal T Cell Expressed (RANTES). (E) Granulocyte-macrophage colony-stimulating factor (GM-CSF). DCtrl, eSF from controls: dPCOS eSF from women with PCOS (8/12) that exhibit decidualization; ndPCOS, eSF from women with PCOS (4/12) that did not decidualize. The significant differences (P < 0.05) are marked as follows: — (line) between the different treatment groups within the study group (dCtrl or dPCOS or ndPCOS) and * (asterisk) compared with dCtrl and † (cross) compared with dPCOS in the same treatment group between different study groups.
increased IL-8 levels compared with E₂ treatment, although in ndPCOS IL-8 levels were increased compared with dCtrl and dPCOS. The increase in IL-8 may partly explain the chemoattractant profile of ndPCOS media in the migration assays and may relate to imbalanced leukocyte migration that has been suggested to be related to implantation failure (Tuckerman et al., 2010; Ramos-Medina et al., 2013).

As both IL-6 and 8 were increased in E₂P₄ treated ndPCOS samples, we also tested whether they were able to inhibit decidualization in eSFctrl. After treatment, all samples showed high IGFBP-1 levels and typical morphology for decidualization, suggesting that the high cytokine levels are an adverse outcome of altered cell function in PCOS rather than a cause for the aberrant decidualization per se.

**MCP-1 and RANTES**

Basal levels of other chemoattractants (MCP-1 and RANTES) were also increased in ndPCOS compared with dCtrl and dPCOS. MCP-1 and RANTES are associated with monocyte and activated T-cell chemotraction during implantation, and their actions are tightly linked to the inflammatory cascade and NFκB activity (Zhao et al., 2002; Li et al., 2011). Interestingly, up-regulation of RANTES and MCP-1 and activation of NFκB and MAPK signaling in first trimester decidua have been associated with pre-eclampsia, a condition with increased prevalence in PCOS (Li et al., 2011). MCP-1 may also alter immune cell maturation. Previous studies have reported that Th1 cells (CD4 T-cells) are necessary during the implantation process to promote an immune-tolerant environment. High MCP-1 levels increase terminal differentiation of CD4⁺ cells into Th2 helper cells, which theoretically should improve embryonic tolerance (He et al., 2012). Also, RANTES modulates the immune balance. In *in vivo* studies in mice the placental RANTES expression is tightly regulated by P₄ and high levels lead to embryo resorption (Ramhorst et al., 2007). That E₂P₄ treatment stimulated high levels of RANTES secreted by ndPCOS compared with dCtrl and dPCOS may imply impaired inhibitory P₄ action on RANTES regulation in ndPCOS. This finding is similar to that seen in endometriosis, a condition also presenting with P₄-resistance and subfertility (Hornung et al., 2001; Wieser et al., 2005).

**GM-CSF**

GM-CSF is a chemoattractant for migratory dendritic cells (DCs) and macrophages in endometrium and also promotes their maturation (Robertson et al., 1999; Moldenhauer et al., 2010). Interestingly, GM-CSF-null mice have reproductive defects with increased prevalence of fetal loss (Robertson et al., 2000). That GM-CSF was down-regulated basally in dPCOS versus dCtrl may relate to poor endometrial receptivity and previous findings of altered DC migration in these women (Matteo et al., 2010). However, as the hormone response patterns were similar in all groups, even though GM-CSF levels in ndPCOS tended to be higher compared with other groups, the clinical significance remains uncertain and warrants additional functional studies in PCOS endometrium. Altogether, high chemoattractant cytokine secretion in ndPCOS is consistent with our *in vitro* findings that ndPCOS condition media possess high chemoattractant properties for migratory immune cells.

**MMPs**

MMPs comprise a family of zinc-containing endopeptidases that degrade extracellular matrix, crucial for tissue growth and expansion and blood vessel development (Visse and Nagase, 2003). MMPs are generally involved in implantation as well as in the pathogenesis of endometrial cancer (Caballero-Campo et al., 2002; Ewington et al., 2012). In this study P₄ cytokine secretion aberrancy also in the decidualized PCOS samples. On the other hand, ndPCOS with blunted decidualization presented with increased basal IL-6 secretion and in response to steroid hormones, versus dCtrl and dPCOS. We have recently shown that PCOS women with hyperandrogenism and oligo/amenorrhea have increased IL-6 secretion in proliferative phase endometrium compared with controls (Piltonen et al., 2013). The data herein may imply that this inherent altered cytokine secretion persists later in secretory phase in the cycle, and if so, it may contribute to altered endometrial immune profile in these women (Piltonen et al., 2013). As eSF communicate with endometrial epithelium and endometrial leukocytes, aberrant production of IL-6 could result in altered paracrine signaling throughout the endometrium, resulting in disrupted signaling in luminal epithelium and affecting the crosstalk at the maternal—fetal interface.

**IL-8**

IL-8 is a potent chemoattractant of migrating immune cells and is involved in implantation as well as in the pathogenesis of endometrial cancer (Caballero-Campo et al., 2002; Ewington et al., 2012). In this study P₄
inhibited by P4 (Henriet et al., 2002), and eSF-derived MMPs are up-regulated upon P4 withdrawal, facilitating tissue shedding (Irwin et al., 1996). Furthermore, epithelial MMP2 and 9 have been implicated in endometrial cancer pathogenesis (Karahan et al., 2007). During the implantation process, MMPs, secreted by the epithelium, stroma and trophoblast cells, participate in this complex process (Cha et al., 2012). Interestingly, endometrium in women with endometriosis has increased MMP2 and 3 expression, reflecting P4 resistance (Chung et al., 2002; Jana et al., 2013), as in this study. Basal MMP2 and 3 levels were increased in ndPCOS but also in response to E2 or E2P4 compared with dCtrl, consistent with a blunted response of P4 despite normal PGR expression and suggesting a post-receptor defect. Increased MMP action has also been linked to pre-eclampsia and implantation abnormalities, supporting the inflammatory data presented herein (Lockwood et al., 2014). Despite these compelling results, further studies with additional MMPs, as well as measurement of TIMPs warrant further investigation in PCOS endometrium to understand more fully how MMPs are involved in the PCOS-related endometrial abnormalities.

Limitations of this study

There are several limitations to our study. First, the sample size (especially in the ndPCOS group) was limited. The limitation of PCOS samples was partly due to the fact that only women fulfilling all Rotterdam criteria (and NIH criteria) (hyperandrogenism, oligo-amenorrhea, PCO) for PCOS were included. Furthermore, due to limited amount of samples, we had to include samples both from proliferative and secretory phase. However, we have previously demonstrated that by the third passage, the cycle phase from which eSF are derived does not influence their response to E2 or P4 (Aghajanova et al., 2009). Also, in this study there were no signs of in vivo E2 exposure (reflected in induced PGR gene expression) or E2P4 exposure (e.g. induced IGFBP-I protein and transcripts), and the patterns of secreted proteins were similar among samples obtained in different cycle phases. As all experiments were conducted beyond passage 3, we included samples from different cycle phases. Another limitation is that not all subjects were on Metformin. As Metformin has anti-inflammatory properties (Morin-Papunen et al., 2003), it is possible that it could affect the cytokine response values. However, our statistical analysis indicated little variability of the dPCOS group average of cytokines with or without those two samples. Furthermore, after excluding these two samples no changes were observed with regard to the secreted proteins, PGR transcript expression (E2 sensitivity) or IGFBP-I transcript expression (E3P4 sensitivity), suggesting that using our endpoints, the use of MetforminTM did not affect the readouts.

Other limitations include that our study focused on only one cell type even though previously, we have shown that freshly isolated endometrial epithelium of women with PCOS also exhibits a pro-inflammatory phenotype (Piltonen et al., 2013). Moreover, as our previous study showed inflammatory crosstalk between stromal cells and epithelial cells (Chen et al., 2013), future studies including several cell types should be undertaken. Lastly due to selection of the absolute anovulatory phenotype of PCOS patients in this study, this led to limited access of PCOS samples in the secretory phase (for the more common oligo-ovulatory PCOS phenotype), thus preventing more rigorous histological analysis under natural P4 action in vitro.
Impaired decidualization in PCOS endometrial cells

Conclusion

In conclusion, the study provides novel in vitro data showing that a subset of women with PCOS have an aberrant decidualization response of their eSF to E2 and P4, with concomitant increased pro-inflammatory cytokine, chemokine and MMP release—creating a microenvironment conducive to recruiting migratory immune cells. These data support the idea that the endometrium of women with PCOS may present a compromised endometrial environment for implantation and also abnormal endometrial function, resulting in sub-optimal implantation, and predisposition to endometrial cancer.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

T.T.P.: Original study design, conducted sample collection, sample preparation and experiments, data analysis and drafted the manuscript, workload equal to J.C.C. J.C.C.: Improved study design, conducted sample preparation and experiments, data analysis and drafted the manuscript, workload equal to T.T.P. M.Kh.: Conducted sample preparation and experiments, data analysis and helped drafting the manuscript. M.Ka.: Conducted sample collection and preparation and helped drafting the manuscript. A.L.: Conducted sample preparation and histology readings and helped improving the manuscript. T.S.: Sample collection. N.T.: Sample collection. H.H.: Anthropometric clinical data query, helped improving the manuscript. L.C.G. Principal investigator, study design, improved the manuscript.

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Conflict of interest

None declared.

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