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Hyperandrogenic Milieu Dysregulates the Expression of Insulin Signaling Factors and Glucose Transporters in the Endometrium of Patients With Polycystic Ovary Syndrome

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Abstract

Purpose: Subfertility associated with polycystic ovary syndrome (PCOS) mainly originates from oligoovulation/anovulation. Although insulin resistance and androgen excess are known to cause PCOS-associated implantation failure, the consequences of PCOS on endometrial homeostasis and pathophysiology have not been comprehensively understood. In this study, we examined whether the pathophysiologic milieu of PCOS intrinsically affects expression profiles of genes related to insulin signaling and facilitative glucose transporters (GLUTs) in the human endometrium and/or during in vitro decidualization. **Study Design:** Seven healthy women with regular menstrual cycles and 13 patients with PCOS were recruited for this study. To mimic the hyperandrogenic or hyperinsulinemic milieu in the endometrium of patient with PCOS (PCOSE) in vitro, human endometrial stromal cells (hESCs) were treated with dihydrotestosterone (DHT) or insulin, respectively. **Results:** In PCOSE, messenger RNA (mRNA) levels of insulin receptor (IR), IR substrate (IRS) 1, and IRS2 were significantly increased. Furthermore, GLUT1 and GLUT12 were aberrantly increased. Chronic exposure to insulin or DHT aberrantly increased IRS1/IRS2 phosphorylation and protein levels of GLUT1 and GLUT12 in hESCs, suggesting that not only hyperinsulinemic but also hyperandrogenic conditions affect insulin signaling and glucose metabolism. The mRNA microarrays demonstrated that DHT dysregulates various gene sets, including cell cycle and glucose metabolism, in hESCs. Furthermore, DHT suppressed the expression of GLUT1 and GLUT12 as well as decidualization markers, IGFBP1 and prolactin, during in vitro decidualization. **Conclusions:** The hyperandrogenic milieu affects gene expression profiles, including gene sets associated with insulin signaling, cell cycle, glucose metabolism, and/or glucose transport, in human endometrium and during in vitro decidualization.

Keywords

endometrium, polycystic ovary syndrome, glucose transporter, androgen, decidualization

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent female endocrine conditions, which substantially compromises the health and reproductive capacity of women. Currently, the prevalence of PCOS ranges widely from 4% to 21% in reproductive-age women.[1] Although the pathophysiology of the classic PCOS phenotype is primarily characterized by overexpression of cytochrome P450c17,[2,3] excessive luteinizing hormone (LH) stimulation and hyperinsulinemia are considered cofactors in the hyperandrogenic milieu of PCOS.[4] Overall, obesity and/or insulin resistance occur in one- to two-thirds of adult women with PCOS.[4-6]

Impaired reproductive capacity associated with PCOS mainly encompasses ovulatory dysfunction; however, factors related to blastocyst implantation and maintenance of pregnancy may also contribute to infertility with PCOS.[7] In a previous study, more

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than 80% of women with recurrent miscarriages display polycystic ovarian morphology.[8] In addition, higher rates of miscarriage as well as decreased decidual endovascular trophoblast invasion were reported in patients with PCOS.[9,10] Recently, altered expression of genes related to implantation and unexplained infertility was demonstrated in the endometria of women with PCOS (PCOSE) during the implantation window.[11] There is evidence of a role for insulin resistance in the pathogenesis of implantation failure and recurrent pregnancy loss with PCOS.[12,13] In addition, several in vitro studies have reported adverse effects of androgen on endometrial cell growth, as well as on endometrial decidualization and implantation.[14,15] Despite these reports, the mechanisms underlying impaired endometrial receptivity in women with PCOS are still not completely understood.

The human endometrium exhibits steroid-dependent cyclic changes, requiring intensive glucose metabolism and facilitative glucose transporters (GLUTs).[16] A quantitative evaluation of many GLUTs has recently been conducted in endometria without PCOS before and after decidualization[17]; nonetheless, a detailed and comprehensive quantification of GLUTs has not been performed in PCOSE. Humans have 14 different GLUTs[18]; however, the majority of early studies have focused on the expression of GLUT4 and/or GLUT1 in the human endometrium. Reduced expression of GLUT4, an insulin-sensitive transporter, was the indication of defective insulin signaling in PCOSE.[19-24] These findings led to the idea that the uterine endometrium, like muscle and fat, is an insulin-sensitive tissue. However, other studies have failed to demonstrate detectable levels of GLUT4 in the human endometrium, particularly in human endometrial stromal cells (hESCs).[17,20]

Studies using hESCs revealed a critical role of GLUT1, a ubiquitous noninsulin-dependent GLUT, in decidualization and uterine receptivity.[16,17] The GLUT1 expression significantly increases during ESC decidualization in vitro and is upregulated by progesterone (P_4).[25,26] These findings indicate that proper GLUT1 function is important for decidualization and glucose uptake in these tissues; however, no comprehensive data evaluating the role of other GLUTs and the influence of hyperandrogenic conditions on the decidualization process and uterine receptivity are currently available. Notably, we previously identified a variety of dysregulated signaling pathways, including those related to cell cycle and glucose metabolism, in PCOSE by RNA profiling through a pathway-oriented analysis.[27] Here, we performed further experiments to examine whether the pathophysiologic milieu of PCOS, such as hyperandrogenism, intrinsically affects expression profiles of genes related to insulin signaling and GLUTs in human endometrium and/or during in vitro decidualization.

Materials and Methods

Patients

Twenty-five patients were enrolled between July 2013 and November 2014. The patients who lacked some essential hormone data or adequate endometrial specimen were excluded from the study, and 13 patients with PCOS and 7 normally cycling women were included in this study. All procedures used in this study were carried out in accordance with the principles for conducting experiments on human participants outlined in the Declaration of Helsinki. The institutional review board (IRB) of the CHA Bundang Medical Center of CHA University, Gyeonggi-do, Korea (IRB no. BD2011-115D), approved this study. All human participants signed a written informed consent form before participating in the study.

The diagnosis of PCOS was based on the presence of at least 2 of the criteria suggested by the revised 2003 Rotterdam consensus for PCOS, that is, oligoovulation and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries.[5] Control patients had regular menstrual cycles (at 25- to 35-day intervals) with no clinical or biochemical signs of PCOS. Additional inclusion criteria for all patients were an age of 18 to 40 years and no hormonal treatments, including oral contraceptives, for at least 3 months before entering the study. In addition, no patients presented with thyroid dysfunction, hyperprolactinemia, congenital adrenal hyperplasia, Cushing syndrome, or androgen-producing tumors.

Clinical and Biochemical Assessments

Clinical assessment comprised anthropometric measurements, modified Ferriman-Gallwey score calculation, and blood pressure determination. Patients were asked to answer a questionnaire regarding their family history of hypertension, diabetes mellitus, and other heritable endocrine conditions. To assess basal hormone levels, venous blood samples were obtained between 8:00 and 9:00 AM after a 12-hour period of overnight fasting. In control patients, basal hormone levels were determined on cycle days 2 and 3. Hormone panels consisted of prolactin (PRL), thyroid-stimulating hormone, free thyroxine, LH, follicle-stimulating hormone, estradiol (E_2), P_4, 17α-hydroxyprogesterone, testosterone, dehydroepiandrosterone sulfate (DHEAS), and sex hormone–binding globulin (SHBG). To assess glucose metabolism, glucose and insulin levels were measured under fasting and after an oral glucose tolerance test with a 75-g load of glucose.

Endometrial Sampling

Endometrial samples were obtained from the corpus of the uteri using curettage or a Pipelle endometrial aspirator (CooperSurgical, Trumbull, Connecticut) under sterile conditions. The control endometria were biopsied during the proliferative phase, on cycle days 10 to 12, to achieve morphological and physiological resemblance to the PCOSE. All endometrial specimens were diagnosed and dated by routine pathological analysis according to the Noyes criteria.[28] Tissue samples for RNA extraction were snap-frozen in liquid nitrogen and stored at −70°C.

Treatment of hESCs

An hESC line (CRL-4003) was purchased from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were plated in 6-well cell culture plates at 2×10^5 cells/well in Dulbecco's modified Eagle's medium:F12 without phenol red, supplemented with 2% charcoal-stripped fetal bovine serum and 1% antibiotic–antimycotic solution. To mimic hyperandrogenism and hyperinsulinemia in patients with PCOS in vitro, hESCs were treated with 1, 10, or 100 μM dihydrotestosterone (DHT; Sigma-Aldrich, St Louis, Missouri) for 3 to 9 days[29,30] and 10 nM insulin (Sigma-Aldrich) for 8 hours,[31,32] as previously described with some modification, respectively. Control cells received the vehicle, 0.1% dimethyl sulfoxide.

In Vitro Decidualization of hESCs

The hESCs were treated with 0.5 mM N6, 2́-O-dibutyryladenosine cyclic adenosine monophosphate and 1 μM medroxyprogesterone 17-acetate (both Sigma-Aldrich) for 1 to 9 days to induce decidualization in vitro.

RNA Extraction, Reverse Transcription Polymerase Chain Reaction, and Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from human endometrial tissues and hESCs using TRIzol reagent (Life Technologies, San Diego, California), according to the manufacturer's protocols. First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase and RNasin Ribonuclease Inhibitor (both Promega, Madison, Wisconsin). Real-time polymerase chain reaction (PCR) was performed to quantify expression levels, using iQ SYBR Green Supermix (Bio-Rad, Hercules, California) on a Bio-Rad iCycler. To compare transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established and used to calculate the relative abundance of each gene. In all experiments, ribosomal protein L19 (rPL19) was used as a reference. All PCR reactions were performed in duplicate.

Western Blot Analyses

Cells were lysed in 150 μL lysis buffer containing PRO-PREP protein extraction solution (iNtRON, Seongnam, Korea) and 1× phosphatase inhibitor (Roche Applied Science, Indianapolis, Indiana). Lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10 μg/lane), transferred onto nitrocellulose membranes (Bio-Rad), and blocked with 5% nonfat milk (Bio-Rad) in Tris-buffered saline (Bio-Rad) containing 0.1% Tween 20 (Sigma-Aldrich). Membranes were incubated overnight at 4°C with the following antibodies: GLUT1 (1:1000; Cell Signaling Technology, Denver, Colorado); GLUT8 (1:500; Santa Cruz Biotechnology, Santa Cruz, California); GLUT12 (1:1000; Abcam, Cambridge, Massachusetts); phospho-IRS1/2 (Tyr612; 1:1000; Santa Cruz Biotechnology); IRS1 (1:1000; Cell Signaling Technology); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:2000; Cell Signaling Technology). Horseradish peroxidase-conjugated goat-anti-rabbit and mouse immunoglobulin G were diluted to 1:3000 in Tris-buffered saline Tween-20 with 5% milk and used as secondary antibodies (incubation for 1 hour at room temperature [25°C]). The signals were developed using the Clarity ECL Western blotting substrate kit and detected using a ChemiDoc XRS+ system with Image Lab software (version 4.0; all Bio-Rad).

Immunofluorescence Analyses of hESCs

Cells were fixed in 4% paraformaldehyde and incubated with GLUT1 (1:100), GLUT8 (1:100), GLUT12 (1:50), and phospho-IRS1/IRS2 (1:50) primary antibodies at 4°C for 24 hours. Cells were then washed in phosphate-buffered saline and incubated with appropriate secondary antibodies conjugated to fluorescein isothiocyanate (1:200; Jackson ImmunoResearch, West Grove, Pennsylvania). For negative controls, the primary antibodies were omitted, but the cells were incubated with secondary antibodies. Nuclei were counterstained with 4́,6-diamidino-2-phenylindole dihydrochloride. Images were obtained using an Axio Imager 2 microscope with ZEN 2012 software (Carl Zeiss, Jena, Germany).

Microarray and Data Analyses for Expression Profiling With Gene Set Enrichment Analysis

Biotinylated cRNA was prepared from 500 ng total RNA according to the standard Affymetrix protocol (Expression Analysis Technical Manual, 2001; Affymetrix, Santa Clara, California). Following fragmentation, 12 μg of amplified RNA was hybridized for 16 hours at 45°C on GeneChip PrimeView human gene expression arrays (Affymetrix), which contain approximately 20 000 well-characterized human genes, at the Biocore facility (Seoul, Korea). Fold-change filters included the requirement that the genes be present in at least 200% of controls for upregulated genes and less than 50% of controls for downregulated genes.

Expression values and detection calls were computed from the raw microarray data, and GSEA version 4.0 (Broad Institute, Cambridge, Massachusetts) was applied to interpret expression profiles.[33] Pathways were ranked according to the significance of enrichment, and the validation mode measure of significance was used to identify pathways of greatest enrichment. Significance was tested by comparing the observed enrichment with that in data sets with randomly permutated sample labels ($n = 1000$). Gene sets consisting of less than 15 or more than 500 genes were filtered out by gene set size filters.

Statistical Analysis

Groups were compared using unpaired Student t test, Mann-Whitney test, or Wilcoxon signed-rank test. In all cases, P values $<.05$ were considered significant.

Table 1. Comparison of the Clinical Characteristics and Hormonal Data in Patients With PCOS and in Normal Cycling Controls.[a]

Parameters	PCOS, n = 13	Control, n = 7	*P* Values
Age (years)	27.4 (5.3)	34.4 (2.0)	.007
BMI (kg/m^2)	24.9 (5.2)	23.3 (4.1)	.605
E_2 (pg/mL)	58.9 (34.8)	35.3 (13.4)	.052
FSH (mIU/mL)	6.61 (2.34)	6.96 (1.13)	.782
LH (mIU/mL)	12.18 (5.80)	3.96 (1.43)	.001
DHEAS (μg/dL)	235.4 (99.3)	174.1 (75.4)	.191
Testosterone (ng/mL)	0.64 (0.30)	0.26 (0.17)	.003
SHBG (nmol/L)	37.1 (15.1)	69.2 (39.8)	.075
Insulin 0 minute (μU/mL)	9.58 (6.75)	3.84 (2.95)	.036
Insulin 120 minutes (μU/mL)	69.4 (74.6)	13.1 (8.5)	.024
Glucose 0 minute (mg/dL)	99.5 (6.0)	100.3 (5.3)	.499
Glucose 120 minutes (mg/dL)	117.3 (30.3)	108.9 (17.0)	.500

Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; E_2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin.
[a]Data expressed as the mean (standard deviation).

Table 2. Comparison of Clinical and Hormonal Characteristics in Lean (Lean PCOS) and Overweight/Obese (Obese PCOS) Women With PCOS.[a]

Parameters	Lean PCOS, n = 7	Obese PCOS, n = 6	*P* Values
Age (years)	27.3 (5.6)	27.5 (5.3)	.943
BMI (kg/m^2)	20.9 (2.0)	29.7 (3.1)	.003
E_2 (pg/mL)	64.0 (42.8)	53.0 (25.1)	.568
FSH (mIU/mL)	6.97 (2.66)	6.18 (2.05)	.475
LH (mIU/mL)	14.88 (4.94)	9.02 (5.41)	.063
DHEAS (μg/dL)	257.9 (58.9)	209.3 (134.1)	.253
Testosterone (ng/mL)	0.72 (0.36)	0.55 (0.19)	.317
SHBG (nmol/L)	49.0 (7.8)	23.1 (6.3)	.003
Insulin 0 minute (μU/mL)	4.37 (1.40)	15.67 (4.94)	.003
Insulin 120 minutes (μU/mL)	17.4 (7.8)	130.1 (71.2)	.003
Glucose 0 minute (mg/dL)	100.3 (5.0)	98.7 (7.3)	.389
Glucose 120 minutes (mg/dL)	96.4 (17.2)	141.7 (23.1)	.004

Abbreviations: BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; E_2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PCOS, polycystic ovary syndrome; SHBG, sex hormone-binding globulin.
[a]Data expressed as the mean (standard deviation).

Results

Clinical Characteristics and Hormonal Profiles of Patients With PCOS

The clinical and hormonal characteristics of all patients are summarized in Table 1. Body mass index (BMI) was similar between control patients and patients with PCOS. Plasma levels of LH ($P = .001$) and testosterone ($P = .003$) were significantly higher in patients with PCOS. Meanwhile, plasma levels of SHBG were lower in the PCOS group compared to the control group, although they did not reach statistical significance. Insulin levels, both fasting and 2 hours after glucose load, were significantly higher in patients with PCOS ($P < .05$).

Table 2 shows a comparison of clinical and hormonal characteristics between lean and overweight/obese women within the PCOS group. As expected, the BMI of patients in the overweight/obese PCOS group was significantly higher than in the lean PCOS group ($P = .003$). While plasma levels of LH, testosterone, and DHEAS were not statistically different between them, plasma levels of SHBG were significantly lower in the overweight/obese PCOS group ($P = .003$). Glucose ($P = .004$) and insulin levels ($P = .003$) 2 hours after glucose load were higher in overweight/obese patients than in lean patients.

Aberrant Upregulation of Factors Involved in Insulin Signaling and GLUTs in PCOSE

To investigate whether the expression levels of factors involved in the insulin signaling pathway are altered in PCOSE, real-time RT-PCR was performed for insulin receptor (IR), IRS1, and IRS2. Expression of IR ($P < .01$), IRS1 ($P < .01$), and IRS2 ($P < .05$) messenger RNA (mRNA) was significantly higher in PCOSE than in control patients (Figure 1A). However, there were no significant changes in the mRNA expression of IR, IRS1, and IRS2 between the endometria of lean and overweight/obese patients with PCOS (Figure 1B).

To determine whether mRNA levels of GLUTs are altered in PCOSE, mRNA expression patterns for all GLUTs were examined in PCOSE (PCOS) and control (CON) endometria (Figure 1C). Among them, GLUT1 ($P < .01$) and GLUT12 ($P < .001$) were significantly upregulated in PCOSE compared with that in control patients (Figure 1D). The expression of GLUT8 was also higher in patients with PCOS, but the difference did not reach statistical significance. The mRNA levels of other GLUTs, including GLUT4, were not different between the 2 groups or were very low to undetectable in all endometrial samples (Figure 1C, data not shown). Whereas the levels of GLUT1 and GLUT12 mRNAs were significantly different between endometria of controls and PCOSE, there were no differences between the subgroups of PCOS (Figure 1E). Collectively, these results suggest that aberrant regulation of IR, IRSs, and GLUTs is associated with the pathophysiological conditions of PCOS such as hyperandrogenism rather than others such as obesity and/or insulin resistance.

Exposure to Androgen as well as Insulin Aberrantly Upregulates IRS1/IRS2 Phosphorylation and GLUTs Expression in hESCs In Vitro

To further understand the influences of hyperinsulinemia or hyperandrogenism on the human endometrium, major factor(s) for insulin signaling pathway and GLUTs were examined in insulin- or androgen-treated hESCs, respectively. Immunofluorescence staining showed that phosphorylation of IRS1/IRS2 on Tyr612 was increased in both insulin- and androgen-treated hESCs (Figure 2A). Although total IRS1 after insulin or androgen treatment was similar to the control level, both

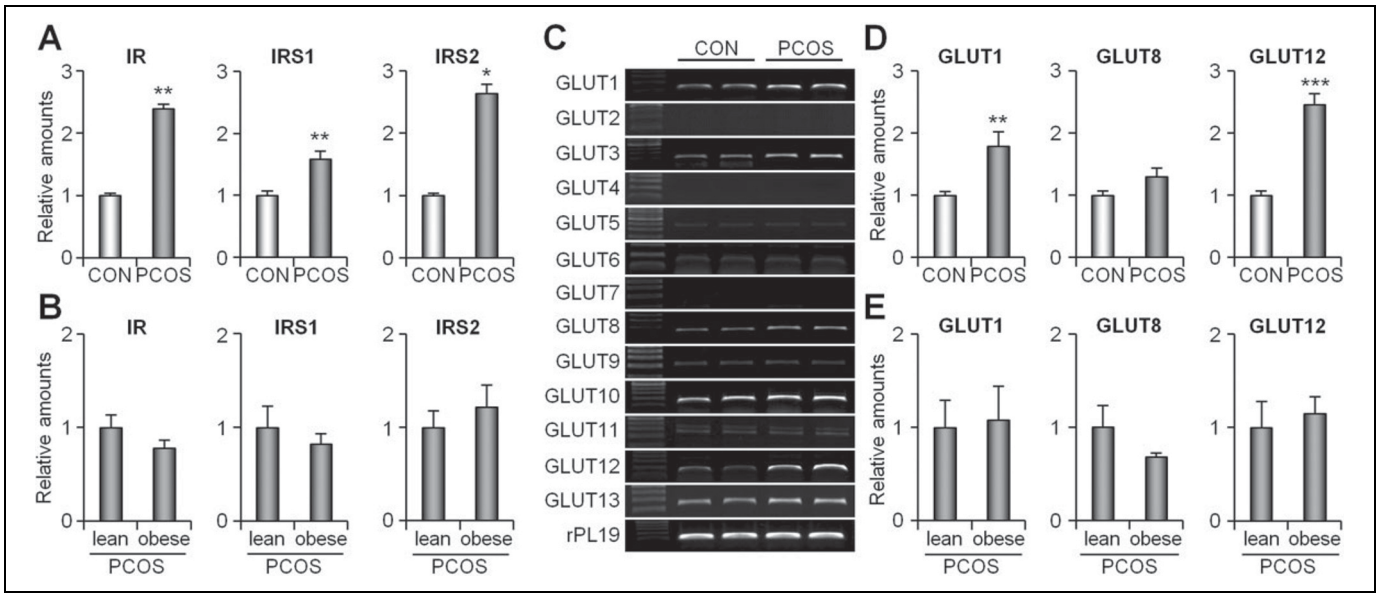

Figure 1. Aberrant upregulation of insulin receptor (IR), IR substrates (IRS1 and IRS2), GLUT1, and GLUT12 in the endometria of patients with PCOS. A and B, Real-time RT-PCR for IR, IRS1, and IRS2 mRNA expression between endometria of healthy women (CON) and patients with PCOS (PCOS) (A) and between endometria of lean and overweight/obese patients with PCOS (B). rPL19 was used as a reference. C, The mRNA expression profiles for all human GLUT family members between endometria of control (CON) and patients with PCOS (PCOS) by RT-PCR. D and E, Real-time RT-PCR of GLUT1, GLUT8, and GLUT12 mRNA between CON and PCOS (D) and between endometria of lean and overweight/obese patients with PCOS (E). rPL19 was used as a reference. *$P < .05$, **$P < .01$, and ***$P < .001$ compared to control patients. GLUT indicates glucose transporter; mRNA, messenger RNA; PCOS, polycystic ovary syndrome; RT-PCR, reverse transcription polymerase chain reaction.

insulin and androgen increased the p-IRS1/IRS2 signal on Tyr612 in cultured hESCs (Figure 2C and D).

Immunofluorescence staining clearly showed that protein levels of GLUT1 and GLUT12 were increased in not only insulin-treated but also androgen-treated hESCs, whereas GLUT8 did not show a significant change (Figure 2B). Western blot analyses reinforced the finding that GLUT1 and GLUT12 are aberrantly increased in ESCs exposed to PCOS-like environments (Figure 2C and D). These data suggest that androgen as well as insulin could disturb the insulin signaling pathway and cellular glucose uptake via aberrant regulation of GLUTs.

Chronic Exposure to Androgen Systemically Alters Genome-Wide Expression Profiles of hESCs

To examine how androgen affects the homeostasis of hESCs, the genome-wide expression profiles of hESCs cultured with DHT (DHT) or without DHT (CON) were compared. Unsupervised hierarchical clustering demonstrated that global expression patterns were distinctly different between CON and DHT (Figure 3A). Heat maps in Figure 3B represent the expression of the 50 most increased and decreased genes in DHT. Many genes involved in lipid metabolism and/or prostaglandin synthesis, such as *ADH1B*, *PTGIS, FABP4*, *ATP8B4*, *PTGES*, and *PLA1A*, were included in the list of the 50 most increased genes in DHT. Furthermore, the genes induced by interferon(s) for immune responses, such as *OAS1*, *OAS2*, *IFI27*, *IFI44L*, *IFI6*, and *IFIH1*, were upregulated by androgen treatment. Among the genes most decreased in DHT were a variety of cell-cycle regulators, such as *IGF2*, *E2F7*, *MCM10*, *CDC45*, *E2F8*, *CCNE2*, *CDC25A*, *CDC6*, and *CDK1*.

To understand the mechanisms through which androgen affects hESC homeostasis, it is critical to identify the significantly dysregulated signaling pathways and biological processes. Supervised analyses, such as GSEA, provide such insights (Supplementary Table 1), as well as a list of differentially expressed genes (Figure 3B). Androgen treatment yielded 604 and 687 gene sets enriched in DHT and CON with a false discovery rate of 25%, respectively. Supplementary Table 1 includes selective lists from enriched gene sets of DHT and CON. The table shows that gene sets associated with glucose metabolism, cell cycle, estrogen receptor α targets, STAT3 targets, and inflammation were dysregulated by androgen treatment. For example, "glycolysis-gluconeogenesis," "transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds," and "IRS1 targets down" gene sets were systemically enriched in DHT, whereas "glucose transport," "regulation of glucokinase by glucokinase regulatory protein," and "glucagon signaling in metabolic regulation" were downregulated by androgen treatment. Figure 3C presents heat maps for cell cycle and glycolysis_gluconeogenesis as representative gene sets enriched in CON. The RT-PCR results showed downregulation of genes encoding cell cycle regulators and upregulation of genes related to glycolysis-gluconeogenesis (Figure 3D). These results suggest that

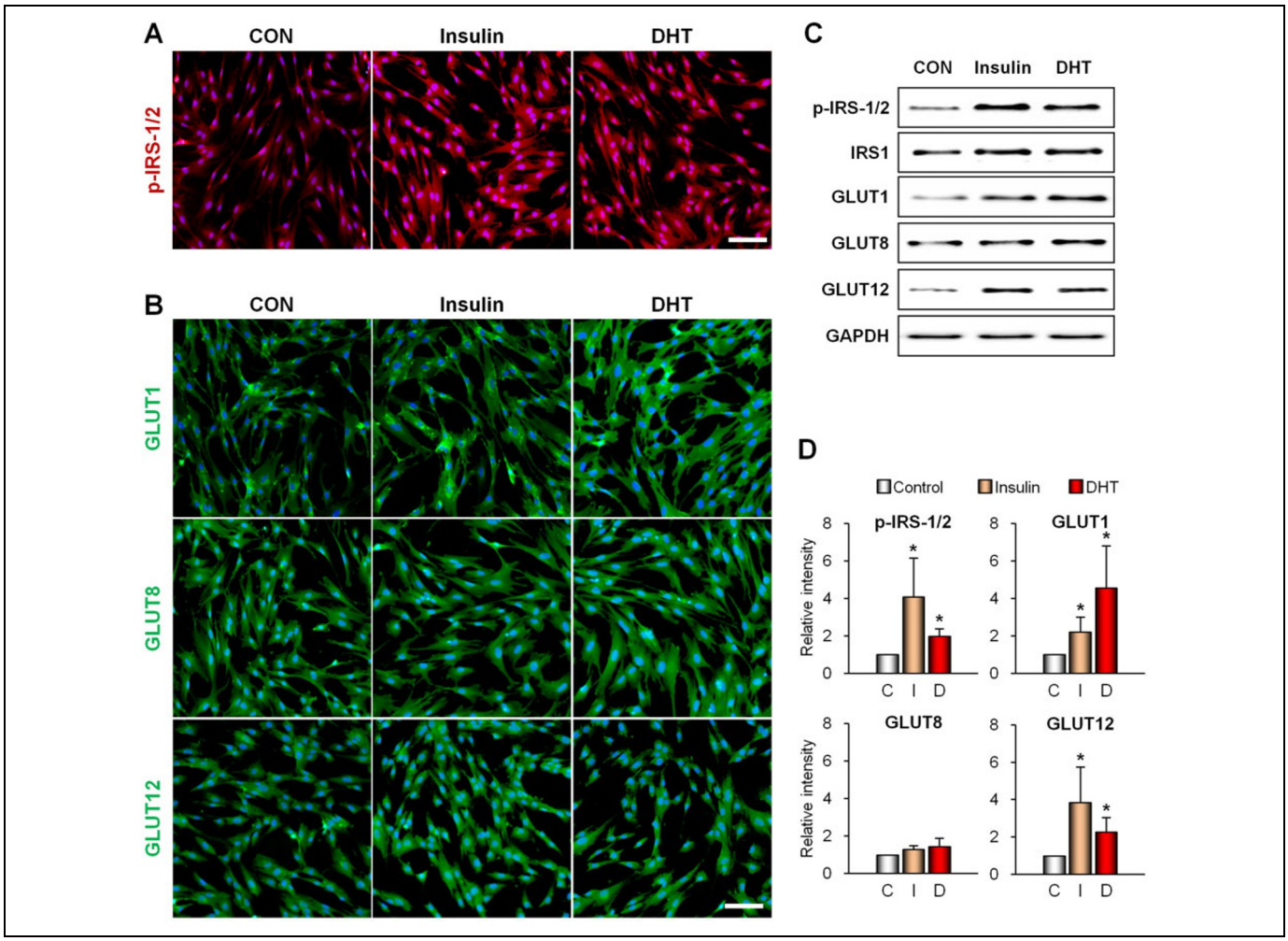

Figure 2. Immunofluorescence and Western blotting analyses of IRS1/IRS2 and GLUT1, GLUT8, and GLUT12 in hESCs treated with insulin or DHT. A, Immunofluorescence staining for p-IRS1/IRS2 in hESCs treated with either insulin or DHT. B, Immunofluorescence of GLUT1, GLUT8, and GLUT12 in hESCs. Immunoreactive signals were either green or red, and nuclei were counterstained with DAPI. C and D, Western blotting for phosphorylation status of IRS1/IRS2 and GLUTs in hESCs treated with insulin or DHT. GAPDH was used as a loading control for Western blotting analyses. The intensity of the protein band was normalized with GAPDH band intensity in each corresponding land. Scale bar: 100 μm. CON, vehicle-treated hESCs; Insulin, insulin-treated hESCs (10 nM); DHT, DHT-treated hESCs (1 μM). *$P < .05$ compared to control samples. DAPI indicates 4′,6-diamidino-2-phenylindole dihydrochloride; DHT, dihydrotestosterone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; hESCs, human endometrial stromal cells; IRS, insulin receptor substrates.

glucose transport and/or metabolism are dysregulated in hESCs by chronic androgen treatment, as is cell cycle regulation. Interestingly, gene sets known to be regulated by androgen signaling were not included in the list of dysregulated gene sets in DHT.

DHT Suppresses the Gradually Increased Expression of GLUT1 and GLUT12 During In Vitro Decidualization

Since there were no differences in insulin signaling between lean and overweight/obese patients with PCOS (Figure 1A and B), we hypothesized that the hyperandrogenic environment in PCOS may play a major role in uterine physiology, including glucose metabolism and decidualization. During in vitro decidualization, DHT significantly and dose-dependently decreased the mRNA expression of PRL and IGFBP1, well-known decidualization markers (Figure 4A). Inhibitory effects of androgen on hESC decidualization were also noted upon histologic observation of cultured hESCs (Figure 4B). The PRL and IGFBP1 mRNA levels, suppressed by chronic DHT treatment, were partially restored by flutamide, an androgen receptor antagonist (Figure 4C), suggesting that the androgen signaling pathway could affect stromal cell decidualization for embryo implantation. Among the 5 GLUTs that were expressed in hESCs, the expression levels of GLUT1, GLUT8, and GLUT12 were gradually increased during in vitro decidualization (Figure 4D). In contrast to undecidualized endometria from patients with PCOS (Figure 1C-E), DHT treatment significantly and dose-dependently reduced the expression of GLUT1 and GLUT12 mRNAs in hESCs during in vitro

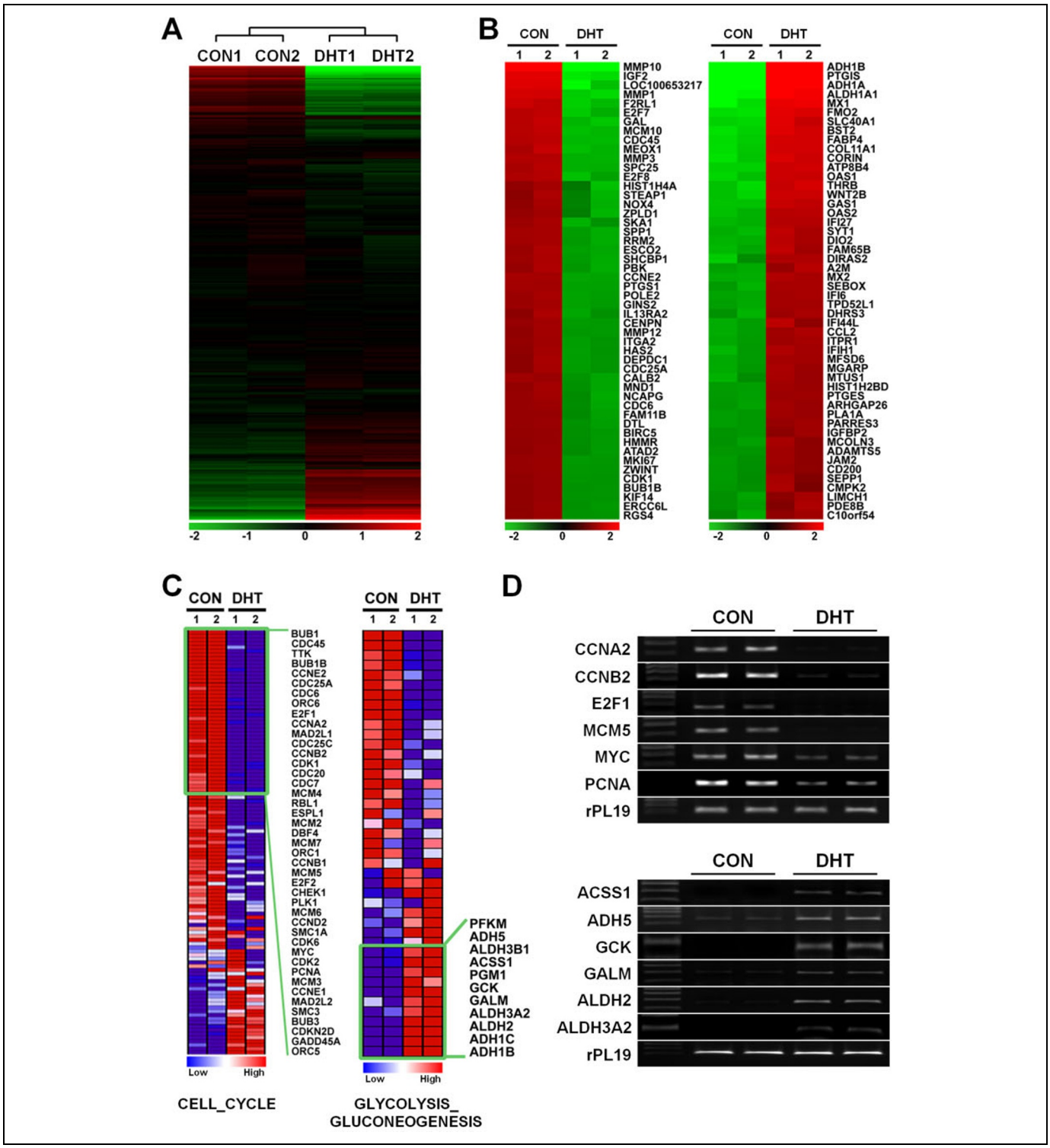

Figure 3. Genome-wide expression profiles of hESCs chronically exposed to androgen. A, Unsupervised hierarchical clustering analysis for mRNA microarray data of hESCs exposed either vehicle (CON) or androgen (DHT). B, Heat maps indicating the expression of the top 50 significantly increased and decreased genes in DHT-treated hESCs. C, Representative gene sets enriched in control (CON) and DHT-treated ESCs (DHT), cell_cycle and glycolysis_gluconeogenesis, respectively. Genes within the green boxes are leading characters for building enrichment scores in CON or DHT. D, The RT-PCR results for a sample of genes from the green boxes of representative gene sets that either were downregulated or upregulated in DHT-treated hESCs. rPL19 was used as a reference. The color spectrum from green (or blue) to red indicates low to high expression. DHT indicates dihydrotestosterone; hESCs, human endometrial stromal cells; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction.

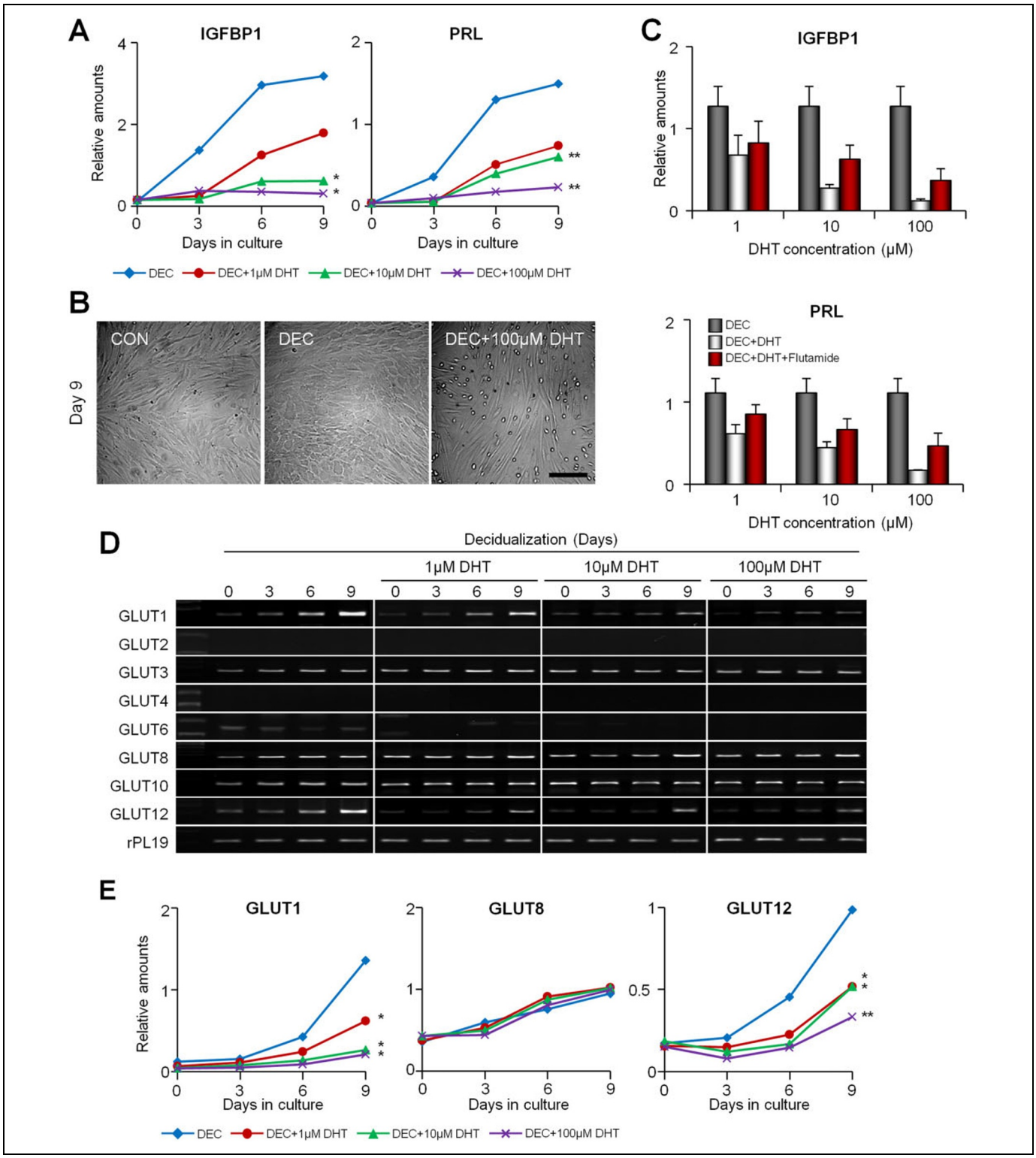

Figure 4. Negative effects of androgen on DEC of hESCs in vitro. A, Results of real-time RT-PCR conducted to analyze expression patterns of decidualization markers, IGFBP1, and prolactin (PRL) during in vitro DEC with various concentrations of DHT. rPL19 was used as a reference. B, Light microscopic images of hESCs that underwent DEC in vitro for 9 days with or without DHT. Note that morphological transformation of uterine stromal cells into epithelial-like decidualizing cells is inhibited by DHT. Scale bar: 100 μm. C, Partial restoration of the negative effects of androgen by flutamide, an androgen receptor antagonist, on IGFBP1 and PRL expression in DHT-treated hESCs during in vitro DEC. D and E, Semiquantitative (D) and real-time RT-PCR analyses (E) of GLUT mRNA levels in hESCs during in vitro DEC with or without DHT. rPL19 was used as a reference. *$P < .05$ and **$P < .01$ compared to control samples. DEC indicates decidualization; DHT, dihydrotestosterone; GLUT, glucose transporter; hESCs, human endometrial stromal cells; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction.

decidualization, whereas GLUT8 expression was not influenced (Figure 4E).

Discussion

Studies have suggested that proper glucose metabolism is an important factor in endometrial differentiation and successful blastocyst implantation.[16,17,25] Cellular uptake of glucose by GLUTs is a critical initial step in glucose utilization. The GLUTs, which are members of the solute carrier 2 (SLC2) family, are characterized by the presence of 12 membrane-spanning helices and several conserved sequence motifs.[16,34] In this study, we investigated all GLUT family members, including those that have been previously identified in the human endometrium.[16] To the best of our knowledge, this is the first work presenting a detailed and comprehensive quantification of a series of GLUTs in the endometria of patients with PCOS.

The GLUT1 is responsible for basal glucose uptake and nutritional support in all cell types, independent of the insulin signaling pathway.[35] Since its first detection in the human endometrium, increased endometrial GLUT1 expression has been reported in conditions with high-energy demands, such as decidualization and endometrial adenocarcinoma.[17,25,26,36-39] Considering this, we suggest that the increased energy demands caused by chronic stimuli from hyperestrogenic and hyperinsulinemic conditions in patients with PCOS might contribute to increased GLUT1 expression in the endometrium (Figure 1D). Further studies are required to explore the underlying molecular mechanism(s) by which GLUT1 is increased in PCOSE.

The GLUT4 is a well-studied GLUT expressed in insulin-sensitive tissues, such as striated muscle and adipose tissue. Many studies have reported reduced levels of GLUT4 and IRS1 mRNA in PCOSE.[19-24,40,41] Decreased IRS1 protein expression upon testosterone treatment was reported in ex vivo cultured endometrial epithelial cells.[41] However, GLUT4 mRNA was not detected in the endometria of either patients with PCOS or control patients in this study (Figure 1C). This is consistent with other studies reporting undetectable levels of GLUT4 in hESCs and ESCs.[17,20,25] For example, Mozzanega et al reported that they failed to detect GLUT4 expression in the stromal cells, whereas a decreased but detectable level of GLUT4 expression was noticeable in epithelial cells from PCOSE.[20] It is still controversial whether GLUT4 is expressed in cyclic human endometrium.

We observed increased expression of IR, IRS1, and IRS2 mRNAs in PCOSE (Figure 1A). This is in contrast with previous reports of reduced IRS1 mRNA in PCOSE.[40,41] However, in vitro analyses using hESCs reinforced our in vivo data from PCOSE. It is well known that Tyr612 is important for IRS1 to activate the PI3K-dependent pathway and ultimately mediate the translocation of GLUT4 in response to insulin stimulation.[42] Tyrosine phosphorylation of IRS1 plays a positive role in insulin signaling. In this study, we observed an increase in the phosphorylation levels of IRS1/IRS2 on Tyr612 in androgen-treated hESCs, suggesting that not only hyperinsulinemic but also hyperandrogenic condition increases the phosphorylation of IRS1/IRS2 in human endometrium.

We also found an increased level of GLUT12 mRNA in PCOSE. The GLUT12 is a recently discovered member of the SLC2 family and has been demonstrated to be insulin sensitive in human muscle cells and transgenic mice.[43,44] Considering that insulin resistance is an innate feature of PCOS, the underlying mechanism for increased GLUT12 expression in the PCOSE remains unknown. Interestingly, a previous study demonstrated that insulin increased the translocation of GLUT4 but not GLUT12 in healthy myometrial cells.[45] In addition, they observed increased cell surface expression of GLUT12 in diabetic myometrium, potentially as a compensatory mechanism for the observed downregulation of GLUT4. It would be quite interesting to examine any cooperative and/or balanced actions of GLUT4 and GLUT12 in insulin sensitivity in the human endometrium.

Similar to several previous studies,[17,25,26] we demonstrated increased expression of GLUT1 during in vitro decidualization (Figure 4D and E). Notably, we also demonstrated increased expression of GLUT12 mRNA during the in vitro decidualization process (Figure 4E). These results suggest a possibility that like GLUT1, GLUT12 may function to transport glucose in an insulin-independent manner in the human endometrium. Remarkably, DHT treatment inhibited increased expression of GLUT1 and GLUT12 in decidualizing hESCs in this study, suggesting that a hyperandrogenic environment might adversely affect uterine receptivity via reduced decidualization in PCOSE. It is supported by previous studies that knockdown of GLUT1 mRNA in ESCs is associated with impaired decidualization.[17] Collectively, aberrant regulation of insulin-dependent and/or insulin-independent glucose uptake by hyperandrogenism could alter environments for embryo implantation in the PCOSE. However, Kajihara and colleagues suggested that DHT at 0.1 or 1 μM provides positive effects on in vitro decidualization of primary hESCs by promoting resistance to oxidative stress.[46,47] The discrepancy of DHT effects on in vitro decidualization of hESCs could be derived from different natures between primary and transformed ESCs. Further studies are strongly needed to understand exact functions of androgen on endometrial physiology in humans.

We investigated the genome-wide expression profiles of ESCs cultured with an androgen (DHT) or a vehicle control (CON). According to the selective lists of gene sets that were enriched in DHT and CON, gene sets associated with glucose metabolism, cell cycle, estrogen receptor α targets, STAT3 targets, and inflammation were dysregulated by androgen treatments (Supplementary Table 1); some of these results are in agreement with those of a previous study of PCOS-affected endometria.[27] Semiquantitative RT-PCR analysis in this study demonstrated that proteins imperative for cell-cycle progression, including cyclins (CCNA2 and CCNB2), minichromosome maintenance proteins (MCM5), and the transcription factor (E2F1), are collectively downregulated in DHT (Figure 3D). These results suggest that decreased expression of cell-cycle regulators in the PCOSE might be related to

hyperandrogenism rather than hyperinsulinemia. However, it is interesting to mention that gene sets either upregulated or downregulated by androgen were not included in the list of gene sets enriched in DHT. Androgen significantly disturbed genome-wide profile of gene expression (Figure 3) but not specific gene sets directly regulated by androgen during culture (data not shown). This suggests that chronic and acute exposure to DHT may alter different sets of genes during regular culture and/or in vitro decidualization of ESCs. Furthermore, it could be due to concentrations of DHT, duration of chronic exposure to androgen, and/or different natures of endometrial cells used in studies. Although these data demonstrate the influence of a hyperandrogenic condition on the insulin signaling pathway and GLUTs in the human endometrium, the underlying mechanisms through which a hyperandrogenic environment influences glucose metabolism in nonpregnant versus decidualized endometrium need to be further explored.

Authors' Note

MHL, JAY, BSL, HS, and DHC designed the study; MHL, JAY, HS, and DHC conducted the experiments; MHL, JAY, BSL, HS, and DHC analyzed the data; HRK, YSK, SWL, and BSL interpreted data; MHL, JAY, HS, and DHC wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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