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THE GLYCOSYLTRANSFERASE EOGT REGULATES ADROPIN EXPRESSION
IN DECIDUALIZING HUMAN ENDOMETRIUM

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Abstract

In pregnancy, resistance of endometrial decidual cells to stress signals is critical for the integrity of the feto-maternal interface and, by extension, survival of the conceptus. O-GlcNAcylation is an essential post-translational modification that links glucose sensing to cellular stress resistance. Unexpectedly, decidualization of primary endometrial stromal cells (EnSCs) was associated with a 60% reduction in O-GlcNAc modified proteins, reflecting downregulation of the enzyme that adds O-GlcNAc to substrates (O-GlcNAc transferase, OGT) but not the enzyme that removes the modification (O-GlcNAcase, OGA). Notably, EOGT, an endoplasmic reticulum-specific O-GlcNAc transferase that modifies a limited number of secreted and membrane proteins, was markedly induced in differentiating EnSCs. Knockdown of EOGT perturbed a network of decidual genes involved in multiple cellular functions. The most downregulated gene upon EOGT knockdown in decidualizing cells was ENHO, which encodes adropin, a metabolic hormone involved in energy homeostasis and glucose and fatty acid metabolism. Analysis of mid-luteal endometrial biopsies revealed an inverse correlation between endometrial EOGT and ENHO expression and body mass index. Taken together, our findings reveal that obesity impairs the EOGT-adropin axis in decidual cells, which in turn points towards a novel mechanistic link between metabolic disorders and adverse pregnancy outcome.

Précis

Induction of EOGT, a selective glycosyltransferase downstream of the nutrient-dependent hexosamine biosynthetic pathway, is essential for endometrial decidualization and adropin expression.
Introduction

During the mid-luteal phase of the menstrual cycle, the endometrium becomes transiently poised to transit from a cycling into a semi-permanent tissue that is maintained throughout pregnancy (1). During this window, the luminal endometrial epithelial cells acquire a receptive phenotype and the underlying stromal cells start to differentiate into secretory decidual cells. After breaching of the luminal epithelium, migratory decidual cells rapidly encapsulate the implanting embryo (2), and form a nutritive and immune-privileged matrix that enables trophoblast invasion and placenta formation (3). Once the process of interstitial and endovascular trophoblast invasion begins, the placental-maternal interface is intensely remodeled and exposed to profound changes fluctuations in oxygen tension associated with changes to the vascular tree (1,4). Decidual cells are programmed to resist a range of stressors, thus ensuring integrity of the interface and survival of the conceptus. Several molecular mechanisms underpin this quasi-autonomous state of decidua cells, including silencing of circadian gene expression (5), inhibition of stress pathways such as c-Jun N-terminal kinase (JNK) (4,6), attenuated inositol trisphosphate signaling (7), global cellular hypoSUMOylation (8), resistance to miRNA-mediated gene silencing through loss of argonaute proteins (9), and marked upregulation of free radical scavengers (10).

Posttranslational modification of proteins with O-linked β-N-acetylglucosamine (O-GlcNAc) is an integral component of the cellular stress response (11,12). O-GlcNAcylation refers to the covalent addition of a GlcNAc sugar moiety to hydroxyl groups of serine and/or threonine residues of cytosolic, nuclear, and mitochondrial proteins. The O-GlcNAc transferase (OGT) transfers the O-GlcNAc moiety from uridine diphosphate (UDP)-GlcNAc to target proteins, whereas O-GlcNAcase (OGA) removes O-GlcNAc from proteins. UDP-GlcNAc is an end product of the nutrient-dependent hexosamine biosynthetic pathway (HBP), a branch pathway in glycolysis. Increased glucose flux through the HBP elevates UDP-GlcNAc
and drives increased cellular O-GlcNAcylation (13,14). OGT targets in excess of 3,000 proteins (15), enabling it to regulate multiple processes, including signal transduction and transcription in a manner akin to - and cooperative with - protein phosphorylation (11,16). Importantly, increased O-GlcNAcylation is important for cell survival in response to a variety of stressors, including osmotic (12,17), oxidative (18), genotoxic (12,19,20), endoplasmic reticulum (ER) (21), and hypoxia/re-oxygenation stress (21,22).

In addition to OGT, a second enzyme has been identified that catalyzes the transfer of GlcNAc from UDP-GlcNAc to epidermal growth factor (EGF) repeats of extracellular proteins was identified (23,24). By contrast to OGT, this glycosyltransferase, termed EGF domain-specific O-linked GlcNAc transferase (EOGT), resides in the ER and targets seemingly only a very limited number of secreted and membrane receptors, including Notch receptors (25-27).

In this study, we examined the expression OGT, OGA and EOGT upon decidual transformation of primary EnSCs. Although increased O-GlcNAcylation has been implicated in stress resistance, decidualization was associated with a marked reduction in O-GlcNAc-modified proteins, reflecting down-regulation of OGT but not OGA. However, EOGT expression in differentiating EnSCs was increased. While the EOGT target proteins in decidual cells remain to be determined, we demonstrate that EOGT knockdown perturbs the expression of numerous genes; most prominently ENHO, which encodes a newly discovered metabolic hormone, adropin, that regulates lipid metabolism, confers insulin sensitivity, and protects against vascular disease (28,29). Finally, we demonstrate that obesity, a major risk factor for reproductive failure, is associated with lower mid-luteal endometrial EOGT and adropin expression.
Methods

Patient selection and endometrial sampling

The study was approved by the NHS National Research Ethics – Hammersmith and Queen Charlotte’s & Chelsea Research Ethics Committee (1997/5065). Endometrial samples were obtained during the luteal phase of an ovulatory, non-hormonally stimulated menstrual cycle using a Wallach Endocell™ sampler, starting from the uterine fundus and moving downward to the internal cervical ostium. Written informed consent was obtained from all participants in accordance with the guidelines in The Declaration of Helsinki 2000. A total of 193 biopsies were used in this study, including 24 fresh endometrial biopsies processed for primary culture. In addition, 112 biopsies stored in RNA later (Sigma-Aldrich) were used to measure mRNA expression and a further 57 snap-frozen and formalin-fixed biopsies were used for Western blot analysis and immunohistochemistry, respectively. All endometrial biopsies were timed between 6 and 10 days after the pre-ovulatory LH surge. Demographic details are summarized in Supplementary Table 1. None of the subjects was on hormonal treatment for at least 3 months prior to the procedure.

Primary cell culture

EnSCs were isolated and established from endometrial tissues as described previously (30). Confluent EnSC monolayers were decidualized in DMEM/F-12 containing 2% DCC-FBS with 0.5 mM 8-bromo-cAMP (Sigma-Aldrich) and 10^{-6} M medroxyprogesterone acetate (MPA; Sigma-Aldrich) to induce a differentiated phenotype. Culture medium was refreshed every 48 hours. All experiments were carried out before the third cell passage.

Transient transfections
Primary EnSCs were transfected with small interfering RNA (siRNA) using the jetPRIME Polyplus transfection kit (VWR International). Undifferentiated EnSCs were transiently transfected with 50 nM EOGT-siGENOME SMARTpool or siGENOME Non-Targeting (NT) siRNA Pool 1 (GE Healthcare). Transfection studies were performed in triplicate and repeated on primary cultures from 4 subjects.

**Real-time quantitative (RTQ)-PCR**

Total RNA was extracted from EnSC cultures using RNA STAT-60 (AMS Biotechnology). Equal amounts of total RNA were treated with DNase and reverse transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN) and the resulting cDNA used as template in qRT-PCR analysis. Detection of gene expression was performed with Power SYBR® Green Master Mix and the 7500 Real Time PCR System. The expression levels of the samples were calculated using the dCt method, incorporating the efficiencies of each primer pair. The variances of input cDNA were normalized against the levels of the *L19* housekeeping gene. All measurements were performed in triplicate. Melting curve analysis confirmed product specificity.

**Western blot analysis**

Protein extracts were prepared by lysing cells in RIPA buffer containing protease inhibitors (cOmplete, Mini, EDTA-free; Roche). Protein yield was quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate. Equal amounts of protein were separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) before wet-transfer onto nitrocellulose membrane. Global O-GlcNAcylation was determined by spotting 10 μg and 2 μg of total protein lysate directly onto nitrocellulose membranes. Nonspecific binding sites were blocked by overnight incubation with 5% non-fat dry milk in Tris-buffered saline with 1% Tween.
(TBS-T; 130 mmol/L NaCl, 20 mmol/L Tris, pH 7.6 and 1% Tween). The antibodies used in this study are listed in Table 1. Protein complexes were visualized with ECL Plus chemiluminescence. Densitometry was performed using Gene Tools software.

**Immunohistochemistry**

Paraffin-embedded, formalin fixed endometrial specimens were immunostained for EOGT using the Novolink polymer detection systems (Leica) as per manufacturer’s instructions. Universal LSAB Plus kits (DAKO) were used as previously described (31) using primary antibodies against EOGT (1:500 dilution) and ENHO (1:200 dilution). Bright-field images were obtained on a Mirax Midi slide scanner and visualized using Panoramic Viewer software for analysis.

**RNA-Sequencing and data analysis**

Total RNA was extracted using RNA-STAT-60 from primary EnSC cultures first transfected with either EOGT or non-targeting (NT) siRNA and then decidualized with 8-br-cAMP and MPA for 4 days. 3 biological repeats were performed to allow for inter-patient variability. RNA quality was analysed on an Agilent 2100 Bioanalyzer. RNA integrity number score for all samples was ≥ 8.0. Transcriptomic maps of paired-end reads were generated using Bowtie-2.2.3, SAMtools 0.1.19, and TopHat 2.0.12 against the University of California, Santa Cruz hg19 reference transcriptome (2014) from the Illumina iGenomes resource using the fr-firststrand setting. Transcript counts were assessed by HTSeq-0.6.1. Transcripts per million (TPM) were calculated as recently described (32). Differential gene expression analysis was performed using DEseq2-1.14.1. Significance was defined as an adjusted P value (q value) of < 0.05 following Benjamini-Hochberg False Discovery Rate correction. Expression data have been submitted to the Gene Expression Omnibus (GEO) repository (accession number:
Gene Ontology (GO) analyses were carried out using DAVID Bioinformatics Resources 6.8 (33,34) and visualized using REVIGO online software (35). GO Term Gene Set Enrichment Analysis (GSEA), was performed using piano R package (36). Briefly, GO ID was extracted for each Ensembl gene ID using biomaRt package in R (37). Gene ID and GO ID were loaded into correct format using loadGSC function and GSEA was performed using the runGSA function. Genes were ranked according to the adjusted $P$ value and log2 Fold Change was used to determine up or downregulated transcripts.

**Statistical Analysis**

*In vitro* experiments were analyzed with the statistical package Graphpad Prism 6. Unpaired Student’s $t$-test and one-way ANOVA with post hoc Tukey’s test were used when appropriate. The association between *EOGT* and *ENHO* mRNA in endometrial biopsies and BMI was analyzed using Pearson’s rank correlation. Statistical significance was assumed when $P < 0.05$
Results

Loss of OGT-dependent O-GlcNAcylation in decidualizing EnSCs

O-GlcNAcylation of target proteins is enhanced in response to diverse stress signals and tissue injury (15). To test if O-GlcNAcylation plays a role in decidualization, primary EnSCs were decidualized with 8-br-cAMP and MPA for 2, 4 or 8 days and the expression of O-GlcNAc processing enzymes, OGT, EO GT and OGA, examined at both mRNA and protein level. Analysis of 4 independent primary cultures demonstrated that decidualization results in downregulation of the canonical O-GlcNAc transferase OGT at both mRNA and protein level (Fig. 1A and 1B), whereas expression of OGA (encoded by MGEA5) was unchanged. In contrast to OGT, expression of EO GT increased significantly upon decidualization. In fact, induction of EO GT was more marked at protein than mRNA level, with levels increasing ~5-fold after 8 days of decidualization (Fig. 1A and 1B). Densitometric analyses of Western blots are shown in Supplementary Fig. 1.

While thousands of OGT substrates have been identified, only a handful of EO GT targets have been described to date (38,39). To determine the impact of decidualization on total cellular O-GlcNAcylation, protein lysates from undifferentiated EnSCs and cells decidualized for 8 days were subjected to dot-blot analysis using a primary antibody directed against serine and threonine residues with attached beta-O-linked GlcNAc. This analysis revealed ~60% reduction in global O-GlcNAcylation in decidual cells (Fig. 1C), reflecting the relative shift to OGA over OGT. Thus, decidualization is associated with decreased OGT-mediated cellular O-GlcNAcylation, yet increased expression of EO GT, a highly selective transferase that targets secreted and membrane-bound proteins (39).

EO GT expression in mid-luteal endometrium.
Mining of the Genotype-Tissue Expression (GTEx) and FANTOM (Functional Annotation of Mammalian Genomes) projects revealed that \textit{EOGT} is highly expressed in the endometrium compared to other tissues \cite{40,41}. Furthermore, analysis of Gene Expression Omnibus (GEO profile ID: 24476716) demonstrated that \textit{EOGT} mRNA levels in cycling endometrium increases sharply upon transition from the early- to mid-secretory endometrium (Fig. 2A). Laser microdissection of glandular endometrial epithelium coupled to RNA-sequencing revealed a transient 3-fold increase in \textit{EOGT} mRNA levels during the mid-luteal phase, coinciding with the putative window of implantation (Fig. 2B) \cite{42}. Immunohistochemistry was performed to assess the spatiotemporal expression of \textit{EOGT} in the endometrial stromal compartment. In timed early-secretory phase (LH+5) biopsies, \textit{EOGT} immunoreactivity was largely confined to endometrial glands (Fig. 2C, upper panel). During the mid-luteal implantation window (LH+9), stromal cells were strongly \textit{EOGT} positive (Fig. 2C, lower panel). Interestingly, \textit{EOGT} was also expressed in endothelial cells lining the emerging terminal spiral arteries, although the surrounding perivascular cells often appeared devoid of this glycosyltransferase. Thus, \textit{EOGT} is expressed in the endometrial epithelial compartment, decidualizing stromal cells and vascular endothelial cells at the time of embryo implantation.

**Impact of \textit{EOGT} knockdown on decidual marker genes and Notch signaling.**

Induction of decidual marker genes, such as \textit{PRL} and \textit{IGFBP1}, in response to cAMP and progestin signaling is mediated, at least in part, by the auto/paracrine actions of a host of cytokines and morphogens \cite{1}. We speculated that the strong induction of \textit{EOGT} could be essential for the expression of decidual marker genes in differentiating EnSCs. To test this conjecture, 4 primary cultures were first transfected with non-targeting (NT) or \textit{EOGT} siRNA and then decidualized with 8-br-cAMP and MPA for 2, 4 or 8 days. Although \textit{EOGT} knockdown was highly efficient (Fig. 3A, upper panel), there was no significant impact on the
induction of either PRL or IGFBP1 in decidualizing cultures (Fig. 3A, middle and lower panels, respectively).

Notch receptors are perhaps the best characterized targets of EOGT (27). O-GlcNAc modification of epidermal growth factor-like repeats of NOTCH1 enhances signaling by potentiating interaction with Delta like-1 (DLL1) and DLL4 ligands in a cell-specific context (27). Ligand binding to the extracellular domain of Notch receptors induces proteolytic cleavage and releases Notch intracellular domain (ICD), which enters the cell nucleus to regulate gene expression. To test if induction of EOGT in decidualizing EnSCs modulates Notch signaling, total protein lysates of undifferentiated cells and cells treated with 8-br-cAMP and MPA for 2, 4 or 8 days were subjected to Western blot analysis for NOTCH1 and NOTCH3 ICDs. As shown in Figure 3B, decidualization was associated with gradual silencing of Notch signaling and EOGT knockdown had no discernable effect on this response. Further, expression of HEY1 and HES1, target genes of the canonical Notch signaling pathway (43), was not significantly altered upon EOGT knockdown in EnSCs decidualized for 4 days (P > 0.05; Fig. 3C). Taken together, these observations indicate that Notch activity is not likely regulated by EOGT-mediated O-GlcNAcylation in decidualizing cells.

EOGT knockdown perturbs decidual gene expression.

To gain insight in the role of EOGT in decidual cells, total RNA harvested from 3 independent cultures, first transfected with either EOGT or NT siRNA and then treated with 8-br-cAMP and MPA for 4 days, was subjected to RNA sequencing. Approximately, 26-36 million paired-end reads were sequenced per sample. After accounting for variation between primary cultures, the impact of EOGT knockdown on decidual gene expression was highly consistent with principal components (PC) 1 and 2 accounting for 52% and 36% of variance in gene expression, respectively (Fig. 4A). Based on q ≤ 0.05, we identified 340 genes that were
significantly altered upon EOGT knockdown (Fig. 4B); of which 178 (52%) were up- and 162 (48%) down-regulated. Several highly induced decidual genes were downregulated significantly upon EOGT knockdown, including LEFTY2 \( (q = 3.13 \times 10^{-4}) \), CDKN1C \( (q = 5.10 \times 10^{-5}) \), GADD45G \( (q = 9.58 \times 10^{-9}) \) and GPX3 \( (q = 7.17 \times 10^{-12}) \) (Supplementary Fig. 2). EOGT knockdown also downregulated ESR1, coding the estrogen receptor alpha, in decidualizing cells \( (q = 1.07 \times 10^{-3}) \). However, the most repressed gene upon EOGT knockdown was ENHO (Energy Homeostasis Associated gene; \(-2.03 \log_2 \) fold-change; \( q = 5.67 \times 10^{-11}) \), coding adropin, a recently discovered peptide hormone implicated in the regulation of energy homeostasis, insulin resistance and lipid metabolism (28). Interestingly, \textit{IL1RL1}, which encodes the IL-33 receptor, is strongly upregulated upon decidualization (44), yet EOGT knockdown amplified induction of this gene in differentiating EnSCs \( (1.8 \log_2 \) fold-change; \( q = 2.31 \times 10^{-17}) \).

Gene ontology (GO) term enrichment analysis, using both DAVID (Fig. 4C) and GSEA (Supplementary Fig. 3), revealed that EOGT knockdown results in upregulation of genes involved - amongst other categories - in cell adhesion, extracellular matrix organization, and signal transduction (Fig 4C, left panel; Supplementary Table 2; Supplementary Fig. 3). Notable GO terms enriched in downregulated genes include oxidative-reductive process, response to estrogen/estradiol, and inflammatory responses (Fig 4C, right panel; Supplementary Table 2; Supplementary Fig. 3). We also annotated genes perturbed upon EOGT knockdown by their disease association. GO analysis yielded a conspicuous association between EOGT-responsive decidual genes and vascular and metabolic disorders, most prominently type 2 diabetes (Fig. 4D).

**Obesity perturbs the endometrial EOGT-adropin axis**
To explore the putative link with metabolic disorders, we measured EOGT transcript level by RTQ-PCR in 112 mid-luteal (LH+7-10) endometrial biopsies. Demographic details are summarized in Supplementary Table 1. Interestingly, endometrial EOGT mRNA levels correlated inversely with body mass index (BMI) (Pearson’s $r = -0.194$, $P = 0.043$; Fig. 5A). By contrast, no association was found between either OGT or OGA mRNA levels and BMI (Supplementary Fig. 4). Western blot analysis of total protein lysates of 48 biopsies (LH+7-9) substantiated the inverse correlation between endometrial EOGT levels and BMI ($r = -0.335$, $P = 0.02$; Fig. 5B), with levels being significantly lower in clinically obese patients compared to control subjects ($P < 0.03$) (Fig. 5C).

Expression of adropin in the endometrium has not yet been reported. As shown in Figure 6A, ENHO mRNA levels increase significantly in primary EnSCs decidualized with 8-br-cAMP and MPA for 4 days, although the level of induction varied markedly between primary cultures (Fig. 6A, Supplementary Fig. 5). Furthermore, immunohistochemistry of serial endometrial sections (LH+8) revealed that the tissue distribution of adropin is indistinguishable from EOGT, characterized by strong expression in glands, differentiating stromal cells, and endothelial but not perivascular cells (Fig. 6B). Furthermore, a strong positive correlation was observed between EOGT and ENHO transcript levels in 112 timed endometrial biopsies ($r = 0.327$, $P = 0.0003$; Fig. 6C); as well as a negative correlation between ENHO mRNA expression and BMI ($r = -0.178$, $P = 0.044$; Fig. 6D).

**Discussion**

Dynamic changes in protein O-GlcNAcylation enable cells to homeostatically balance energy supply and demand by modulating the stability, localization and function of a myriad of proteins (45). Here, we report that decidualizing EnSCs downregulate OGT expression and intracellular O-GlcNAcylation but upregulate the highly selective glycosyltransferase EOGT.
Increased canonical O-GlcNAcylation is a well characterized pro-survival response (15); rendering the downregulation of OGT in differentiating EnSCs counterintuitive, especially as initiation of decidual differentiation coincides with a burst of endogenous reactive oxygen species (ROS) production and release of various inflammatory mediators (46,47). However, SUMO modification of proteins is also dramatically reduced in decidualizing cells and uncoupled from JNK-mediated stress signaling though the induction of MAP kinase phosphatase 1 (MKP1) (48-50). Hence, by silencing selective pathways that converge on the posttranslational modification code of numerous proteins, decidual cells appear to prioritize cellular homeostasis over an adaptive response to stress signals. Further, recent studies have shown that decidualization is critically dependent on glucose utilization via the pentose phosphate pathway (51), suggesting that loss of OGT may be integral to the metabolic reprogramming of the endometrium in preparation of pregnancy.

Induction of EOGT in the endometrium during the mid-luteal phase of the cycle coincides with the window of implantation. At this time, EOGT is expressed in the glandular epithelium, vascular endothelial cells, and stromal cells that are poised to decidualize. Decidualization is characterized by an unfolded protein response that underpins ER expansion and acquisition of a secretory phenotype (52). In fact, multiple secreted factors, including interleukin (IL)-11, leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2), have been implicated in the auto/paracrine propagation of the decidual response (1). However, the identity and role of EOGT target proteins, whether secreted or expressed on the cell surface, in differentiating EnSCs requires further investigation. We showed that Notch signaling is attenuated upon decidualization (53), irrespective of EOGT knockdown. Furthermore, other known EOGT target proteins (26), including thrombospondin (THBS1), peptidase domain containing associated with muscle regeneration 1 (PAMR1), and laminin alpha 5 (LAMA5), are also downregulated upon decidualization, at least at mRNA level (GEO
accession number: GSE104720). Although EOGT knockdown did not significantly impact on 
PRL or IGFBP1 expression in differentiating cells, RNA-sequencing uncovered a robust set of 
EOGT-dependent genes. EOGT knockdown upregulated several genes encoding 
inflammatory mediators, including IL-1β (IL1B) and complement component 3 (C3), but 
downregulated key genes involved in decidual stress defenses, such as GPX3 (coding 
extracellular glutathione peroxidase), GLXR (glutaredoxin) and GADD45G (growth arrest and 
DNA damage inducible gamma) (1). EOGT knockdown also blunted the induction of other 
cardinal decidual genes, including F3 (tissue factor) (1), LEFTY2 (left-right determination 
factor 2, also known as endometrial bleeding-associated factor or EBAF) (54), and CDKNIC 
(cyclin dependent kinase inhibitor 1C, p57kip2) (55). Most strikingly, however, was the 
repression of ENHO upon loss of EOGT. ENHO encodes adropin, a recently discovered 
peptide hormone implicated in energy homeostasis, glucose and fatty acid metabolism, and 
vascular protection (56). Although ENHO is primarily expressed in the liver, pancreas and 
brain (28), we showed that this gene is also induced upon decidualization of human EnSCs, 
although the magnitude of induction varied markedly between primary cultures. We further 
showed a strong positive correlation between EOGT and ENHO transcript levels in whole 
endometrial biopsies; and immunohistochemistry on serial tissue sections revealed that the 
cellular distribution of adropin in mid-luteal endometrium is indistinguishable from EOGT. 

GO analysis revealed a putative association between decidual genes perturbed upon 
EOGT knockdown and metabolic and cardiovascular disorders. To explore this possible link 
further, we measured the transcript levels of the 3 O-GlcNAc processing enzymes in 112 
randomly selected mid-luteal endometrial biopsies from women ranging in BMI from 18 to 42. 
A weak but significant negative correlation was observed between BMI and EOGT mRNA 
levels but not OGT or OGA expression. Western blot analysis confirmed that obesity is
associated with impaired endometrial EOGT expression. Furthermore, endometrial ENHO transcript levels also correlated negatively with BMI.

Obesity increases the risk of a spectrum of pregnancy disorders, including obstetrical syndromes, such as pre-eclampsia, fetal growth restriction and preterm labor (57,58), that are caused by impaired endovascular trophoblast invasion and spiral artery remodeling (59). In the absence of physiological remodeling, these uterine vessels are prone to develop acute atherosis, characterized by changes in lipid metabolism, intravascular inflammation, macrophage infiltration and endothelial cell dysfunction (60). Adropin promotes various indices of vascular health, including increased endothelial cell proliferation, migration, and angiogenesis; and diminishes permeability and apoptosis (61). Although as yet untested, these observations suggest that adequate decidual adropin production may be essential for successful spiral artery remodeling in pregnancy. Notably, low circulating adropin levels have not only been associated with high BMI, insulin resistance, endothelial dysfunction and coronary atherosclerosis but also severe preeclampsia (62,63).

In summary, the shift from OGT to EOGT dominance in decidualizing EnSCs results in intracellular hypo-O-GlcNAcylation whereas glucose utilization through the HBP pathway for modification of selective secreted and/or membrane proteins is likely enhanced. We demonstrate that EOGT upregulation is critical for normal decidual function and identified ENHO as major EOGT-responsive gene. Further, our observation that obesity impairs the EOGT-adropin axis in the endometrium intimates a novel mechanistic pathway that links metabolic disorders to vascular placental pathology and adverse pregnancy outcome.
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**Figure 1.** Regulation of O-GlcNAcylation in decidualizing EnSCs. A) OGT, OGA and EOGT transcript levels were measured in undifferentiated EnSCs and cells decidualized with 8-br-cAMP and MPA for 2, 4 or 8 days. Expression was normalized to L19 mRNA levels and the data show mean ± SEM relative to that in undifferentiated cells in 4 independent primary cultures. Different letters above the error bars indicate groups are significantly different from each other at P < 0.05. Group comparison by ANOVA and post hoc Tukey’s test. B) Total protein lysates from parallel cultures were subjected to Western blotting. β-Actin served as a loading control. C) Representative dot blot of total cellular O-GlcNAcylation from total protein lysates from cells decidualized for 2, 4 or 8 days. Lower panel denotes total cellular O-GlcNAcylation relative to β-actin by densitometry and expressed as arbitrary units (A.U.). Group comparison by ANOVA and post hoc Tukey’s test.

**Figure 2.** EOGT expression in mid-luteal endometrium. A) EOGT transcripts, expressed as arbitrary units (A.U.) in proliferative (P), early secretory (ES), mid-secretory (MS) and late secretory (LS) endometrium. The data were derived from in silico analysis of GDS2052 microarray data. B) Expression of EOGT, in transcripts per million (TPM), in ES, MS and LS endometrial glandular epithelium. The data were derived from in silico analysis of published RNA-Seq data (64). Different letters above the error bars indicate groups are significantly different from each other at P < 0.05. Group comparison by ANOVA and post hoc Tukey’s test. C) EOGT immunohistochemistry of mid-luteal endometrial biopsies obtained 5 and 9 days following the LH surge, representing the early- and mid-luteal phase, respectively. Inset in the upper panel shows no staining upon omission of primary antibody (negative control). Arrows indicate positive staining of endothelial cells. Scale bar = 50μm.
Figure 3. **EOGT knockdown in decidualizing EnSCs.** A) Four independent primary cultures were transfected with either non-targeting siRNA (siNT) or siRNA targeting EOGT (siEOGT). After 24 hours, the cultures remained either undifferentiated or were decidualized for 2, 4 and 8 days. Total mRNA was subjected to RTQ-PCR analysis to determine transcript levels for EOGT (left panel), PRL (middle panel) and IGFBP1 (right panel). Expression was normalized to L19 mRNA levels and data show mean ± SEM fold change relative to undifferentiated EnSCs. **P < 0.01.** Group comparison by Unpaired t test. B) Western blot analysis of EOGT, NOTCH1 and NOTCH3 ICDs levels in total protein lysates from EnSCs first transfected with siNT or siEOGT and then decidualized for the indicated time points. β-Actin served as a loading control. Densitometric analyses of NOTCH1 and NOTCH3 expression are shown in middle and right panels, respectively. C) Transcript expression of HES1 and HEY1, normalized to L19 mRNA, from 3 independent primary EnSC cultures transfected with siNT or siEOGT and decidualized for 4 days. Data are mean ± SEM of three biological replicates. P > 0.05 (unpaired t-test).

Figure 4. **EOGT knockdown perturbs distinct gene networks in EnSCs.** A) Principal Component Analysis of RNA-Seq data from 3 independent primary EnSC cultures first transfected with siNT and siEOGT and then decidualized for 4 days. B) Clustered heatmap of RNA-seq data for differentially expressed transcripts between siEOGT and siNT transfected cultures. C) Semantic clustering of significantly overrepresented GO terms (P < 0.05) of differentially up- and down-regulated genes (left and right panel, respectively) upon EOGT knockdown. Circle size is proportional to the percentage of genes in the GO term whereas the color indicates the log10 p-value. Color key is on the right. D) Word cloud presentation of overrepresented disease associations based on GO terms upon EOGT knockdown in decidualizing EnSCs. Size of word is proportional to log10 q-value.
Figure 5. Obesity perturbs endometrial EOGT expression. A) Pearson’s correlation (r) between normalized EOGT transcript levels in mid-luteal endometrial biopsies and BMI in 112 subjects. B) Pearson’s correlation between normalized EOGT protein expression and BMI in mid-luteal endometrial biopsies from 48 subjects. C) Normalized EOGT protein levels in timed endometrial biopsies obtained from women with BMIs of <25, 25-30 (overweight), and > 30 (obese). * P < 0.05 (unpaired t-test).

Figure 6. Endometrial ENHO expression relates to BMI A) ENHO mRNA levels were measured in undifferentiated EnSCs and cells decidualized with 8-br-cAMP and MPA for 4 days (upper panel). Expression was normalized to L19 mRNA levels and the data show fold-change relative to expression in undifferentiated cells in 6 independent primary cultures ** P < 0.01 (unpaired t-test). Total protein lysates from parallel cultures were subjected to Western blotting (lower panel). β-Actin served as a loading control. B) Immunohistochemistry of EOGT (left panel) and adropin (right panel) on serial tissue sections, demonstrating co-localization of both proteins in mid-luteal endometrium. Inset in the left panel shows no staining upon omission of primary antibody (negative control); arrows indicate EOGT and adropin immunoreactivity in endothelial cells of terminal spiral arteries. Scale bars = 50μm. C) Pearson’s correlation between EOGT and ENHO transcripts in endometrial biopsies from 112 subjects. D) Pearson’s correlation between ENHO mRNA levels in mid-luteal endometrial biopsies and BMI in 112 subjects.
Table 1: Primary Antibodies used throughout the study.

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Figure 1:
Figure 2:
Figure 3:

**A**

![Graph showing expression levels of EOOGT, PRL19, and IGFBP1/L19 over different days of decidualization.](image)

**B**

![Western blots showing expression levels of EOOGT, NOTCH1, NOTCH3, and β-actin over different days of decidualization.](image)

**C**

![Graph showing changes in NOTCH1 and NOTCH3 expression over different days of decidualization.](image)
Figure 4:

A. Principal Component Analysis showing the distribution of siNT and siEOGT samples.

B. Heatmap depicting gene expression levels in log-transformed Z-scores.

C. Semantic space representation of up-regulated genes with specific biological processes identified.

D. Textual keywords indicating disease conditions associated with Type 2 Diabetes.
Figure 5:

A: $r = 0.194$, $P = 0.043^*$

B: $r = 0.335$, $P = 0.020^*$

C: 

BMI $<25$, BMI $25-30$, BMI $>30$
THE GLYCOSYLTRANSFERASE EOGT REGULATES ADROPIN EXPRESSION IN DECIDUALIZING HUMAN ENDOMETRIUM – SUPPLEMENTARY FILES

**Supplementary Table 1**: Demographic details of 193 participating subjects.

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Demographic details of participating subjects in mRNA correlative analysis

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Demographic details of participating subjects in protein correlative analysis

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**Supplementary Table 2:** Enriched GO categories upon EOGT knockdown. Categories in *italic* denote overrepresented GO terms of differentially up-regulated genes. Categories underlined denote overrepresented GO terms of differentially down-regulated genes.

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</table>
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4

GO Term Gene Set Enrichment Analysis (GSEA) for Up-regulated genes

- response to progesterone
- interleukin-33-mediated signaling pathway
- signal transduction
- cell adhesion
- extracellular matrix organization
- protein phosphorylation

GO Term Gene Set Enrichment Analysis (GSEA) for Down-regulated genes

- regulation of cell death
- oxidation-reduction process
- cell redox homeostasis
- regulation of cellular amino acid metabolic process
- negative regulation of MAP kinase activity

Legend:
- −log10 adjusted P
- genes involved in GO Term (%)
Supplementary Figure 5
**Supplementary Figure 1.** Regulation of O-GlcNAcylation in decidualizing EnSCs. Related to Figure 1. Densitometric analyses of OGT, OGA and EOGT protein expression from 4 independent primary cultures relative to β-actin expressed as arbitrary units (A.U.). Different letters above the error bars indicate groups are significantly different from each other at $P < 0.05$. Group comparison by ANOVA and post hoc Tukey’s test.

**Supplementary Figure 2.** EOGT knockdown perturbs distinct gene networks in EnSCs. Related to Figure 4. Relative expression of LEFTY2, CDKN1C, GADD45G and GPX3 expressed as transcripts per million (TPM) in 3 independent primary EnSC cultures transfected with siEOGT and decidualized for 4 days relative to cultures transfected with siNT (dotted line).

**Supplementary Figure 3.** EOGT knockdown perturbs distinct gene networks in EnSCs. Related to Figure 4. Semantic clustering of significantly overrepresented GO terms ($P < 0.05$) of up- and down-regulated genes (left and right panel, respectively) upon EOGT knockdown as ranked by $q$ value by GSEA analysis. Circle size is proportional to the percentage of genes in the GO term whereas the color indicates the log10 p-value. Color key is on the right.

**Supplementary Figure 4.** Obesity perturbs endometrial EOGT expression. Related to Figure 5. Pearson’s correlation (r) between normalized OGT and OGA transcript levels in mid-luteal endometrial biopsies and BMI in 112 subjects.

**Supplementary Figure 5.** Endometrial ENHO expression relates to BMI. Related to Figure 6. Densitometric analysis of adropin protein expression from 3 independent primary cultures relative to to β-actin expressed as arbitrary units (A.U.). Different letters above the error bars
indicate groups are significantly different from each other at $P < 0.05$. Group comparison by ANOVA and post hoc Tukey’s test.