Adiponectin regulates glycogen metabolism at the human fetal–maternal interface

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Abstract

Throughout the entire first trimester of pregnancy, fetal growth is sustained by endometrial secretions, i.e. histiotrophic nutrition. Endometrial stromal cells (EnSCs) accumulate and secrete a variety of nutritive molecules that are absorbed by trophoblastic cells and transmitted to the fetus. Glycogen appears to have a critical role in the early stages of fetal development, since infertile women have low endometrial glycogen levels. However, the molecular mechanisms underlying glycogen metabolism and trafficking at the fetal–maternal interface have not yet been characterized. Among the various factors acting at the fetal–maternal interface, we focused on adiponectin – an adipocyte-secreted cytokine involved in the control of carbohydrate and lipid homeostasis. Our results clearly demonstrated that adiponectin controls glycogen metabolism in EnSCs by (i) increasing glucose transporter 1 expression, (ii) inhibiting glucose catabolism via a decrease in lactate and ATP productions, (iii) increasing glycogen synthesis, (iv) promoting glycogen accumulation via phosphoinositide-3 kinase activation and (v) enhancing glycogen secretion. Furthermore, our results revealed that adiponectin significantly limits glycogen endocytosis by human villous trophoblasts. Lastly, we demonstrated that once glycogen has been endocytosed into placental cells, it is degraded into glucose molecules in lysosomes. Taken as a whole, the present results demonstrate that adiponectin exerts a dual role at the fetal–maternal interface by promoting glycogen synthesis in the endometrium and conversely reducing trophoblastic glycogen uptake. We conclude that adiponectin may be involved in feeding the conceptus during the first trimester of pregnancy by controlling glycogen metabolism in both the uterus and the placenta.

Key Words

- adiponectin
- human endometrium
- human placenta
- histiotrophic nutrition
- glycogen metabolism

Introduction

Efficient nutrient exchange between the mother and the fetus is essential for a successful pregnancy and requires adequate placental development. The human placenta is a transient organ composed of embryo-derived cells called trophoblasts. The syncytiotrophoblast (ST) is a specialized multinuclear syncytium located on the outer layer of placental villi. It is directly derived from villous trophoblast (VT) fusion. The ST is crucial for nutrient and
Adiponectin roles in histiotrophic nutrition

Adiponectin is a 30 kDa protein that is described as an insulin-sensitizing hormone (Yamauchi et al. 2001). More precisely, adiponectin stimulates glucose assimilation and fatty-acid transport in muscle cells (Ceddia et al. 2005, Qiao et al. 2012). Moreover, adiponectin promotes the catabolism of carbohydrates and lipids by enhancing the glycolysis and β-oxidation pathways (Yamauchi et al. 2002, Combs & Marliss 2014). These effects seem to be mediated by the activation of the AMP-activated protein kinase (AMPK) pathway (Yamauchi et al. 2002). Furthermore, adiponectin reportedly exerts anti-inflammatory, anti-angiogenic, anti-atherosclerotic, anti-proliferative and pro-apoptotic roles in various cell types (Yamauchi et al. 2003a, Dieudonne et al. 2006, Duval et al. 2016). These effects are mediated by a variety of pathways, including the phosphoinositide-3 kinase (PI3K), the protein kinase A and the mitogen-activated protein kinase pathways (Ouchi et al. 2000, Yamauchi et al. 2003b, Benaitreau et al. 2009).

Some research results suggest that adiponectin is a key actor of the fetal–maternal interface dialog. It has been reported that the adiponectin receptors, ADIPOR1 and ADIPOR2 (Yamauchi et al. 2003b), are expressed in human endometrium and placenta (Takemura et al. 2006, Benaitreau et al. 2009). Experiments performed in our laboratory clearly demonstrated that adiponectin favors the development of a normal placenta with differentiative and anchoring abilities (Benaitreau et al. 2010a,b). Surprisingly, using human and murine models, we and others have shown that adiponectin downregulates glucose and amino acid transporters in placenta (Caminos et al. 2005, Jones et al. 2010, Rosario et al. 2012, Duval et al. 2016). We also demonstrated that adiponectin downregulates mitochondrial biogenesis but increases energy production in human VTs (Duval et al. 2016). Furthermore, the adiponectin system has a critical role in uterine receptivity. Indeed, endometrial ADIPOR expression is abnormally low in infertile women and is at its highest during the implantation window in fertile women (Takemura et al. 2006, Santos et al. 2012). Furthermore, we recently observed that adiponectin exerts anti-differentiative and anti-invasive effects in human EnSCs (Duval et al. 2017).

Considering the importance of adiponectin in the control of glucose metabolism, we aimed to study the in vitro effect of human recombinant adiponectin on glycogen synthesis and trafficking at the human fetal–maternal interface. To this end, we studied (i) glucose metabolism leading to glycogen accumulation in human EnSCs and (ii) glycogen uptake and use in human VTs. Our results contribute to a better understanding of histiotrophic nutrition in general and adiponectin involvement in this pathway in particular.

Materials and methods

Materials

Dulbecco’s Modified Eagle’s Medium and Ham F-12 Nutrient Mix (DMEM/F12), progesterone, 17β-estradiol, penicillin, streptomycin, DNase type I, EDTA, leupeptin, aprotonin, 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), sodium fluoride (NaF), Compound C, LY294002 and bovine serum albumin (BSA) were purchased.
from Sigma Chemical Co. Fetal calf serum (FCS) was purchased from Gibco (Invitrogen). Recombinant human adiponectin was provided by R&D Systems Europe Ltd. Superscript III RNase H-RT and primers were from Invitrogen, and RNase inhibitor was obtained from AMRESCO (Solon, OH, USA). Nucleosip RNA II kit was obtained from Machery-Nagel (Düren, Germany). Trypsin was provided by Difco Laboratories (Detroit, MI, USA). Collagenase A was obtained from Boehringer (Mannheim, Germany). The suppliers of the various antibodies used in our experiments are described in the corresponding sections below.

**Study population and tissue collection**

Endometrial biopsies were programmed during a monitored natural cycle, 7–9 days after the ovulation surge (LH surge), during the hypothetical implantation window. Patients with a low endometrial volume (<2 cm), submucous fibroma or polyps were excluded. Thirty-two normally cycling women (aged 25–41) undergoing endometrial biopsy for fertility evaluation were recruited into the study. First-trimester human placental tissues (4- to 10-week gestational age) were obtained from 12 healthy pregnant women (aged 18–42) undergoing legal abortions. The present study was approved by the local investigational review board (Comité Consultatif de Protection des Personnes dans la Recherche Médicale, approval reference protocol 01-78). All participants provided their written informed consent prior to tissue sampling.

**EnSC culture**

Human EnSCs were isolated and cultured as previously described (Duval et al. 2017). Isolated EnSCs were characterized by positive staining for vimentin (95% stained positive) and negative staining for pancytokeratin (95% stained negative). Isolated EnSCs are able to differentiate in vitro, when incubated with E2 (10^{-8}M) and P4 (10^{-8}M), as shown by the progressive rise in prolactin secretion during the cell culture period (Duval et al. 2017). After isolation, EnSCs were seeded in 12-well culture plates (1.5 × 10^4 cells per well) and cultured in phenol-red-free DMEM/F12 medium supplemented with streptomycin (10 µg/mL), penicillin (100 U/mL) and FCS (10%) at 37°C in a 5% CO₂ and 95% air atmosphere for 48h. EnSCs were then cultured in differentiation medium (DMEM/F12 with E2 (10^{-8}M), P4 (10^{-8}M), charcoal-stripped FCS (2%), penicillin (10 µg/mL) and streptomycin (100 U/mL)). This differentiation medium was supplemented or not with increasing doses of human recombinant adiponectin (from 25 ng/mL to 1000 ng/mL) for 15 days – the time required to obtain fully differentiated decidual cells (Duval et al. 2017). The culture medium was renewed every 48 h.

**Reverse transcription – quantitative polymerase chain reaction**

EnSCs were seeded in 12-well culture plates (3.5 × 10^4 cells per well). After 3, 8 or 15 days of differentiation (D3, D8, D15), total RNA (0.1 µg) from EnSCs was extracted and reverse-transcribed, as previously described (Machinal-Quelin et al. 2002). Quantitative PCR was performed using the C1000 Thermal Cycler (CFX96 real-time system; BioRad, Hercules) and the primers sets indicated in Table 1. Exon-spanning primers were used to avoid non-specific amplification. RNA samples were treated with DNase to avoid non-specific amplification. The amplification specificity was also determined by measuring the melting temperature (Tm) of each primer. The second derivative maximum method was used to automatically determine the crossing point (Cp) for individual samples. The three reference genes (ribosomal protein L13A (RPL13A), TATA-binding protein (TBP) and β2-microglobulin (B2M)) were chosen as previously described (Benaitreau et al. 2009). For each sample, the concentration ratios (target/three reference mRNA) were calculated using CFX Manager (version 3.0, BioRad). The data were expressed as percentages of the control situation. Calibration curves were log-linear over the quantification range, with correlation coefficients (r^2) >0.99 and efficiencies ranging from 1.8 to 2. The intra-assay variability of duplicate crossing point values never exceeded 0.2 cycles and the inter-assay variability (coefficient of variation) ranged from 1.9 to 4.1% for 8–10 runs of each transcript.

**Immunoblotting**

EnSCs were seeded onto six-well culture plates (2.0 × 10^4 cells/well) and lysed on ice in buffer containing Tris (50 mM), NaCl (120 mM), EDTA (1 mM), Nonidet-P40 (1%), deoxycholate (0.1%), SDS (0.1%), sodium vanadate (1 mM), β-glycerophosphate (30 mM), aprotinin (5 µg/mL), leupeptin (12.5 µg/mL), AEBSF (100 µg/mL) and sodium fluoride (10 mM). Twenty micrograms of proteins were resolved by SDS-PAGE (4–20% acrylamide) and transferred for 150 min...
onto nitrocellulose membranes using a wet transfer method. After transfer, membranes were incubated overnight with the primary antibodies described in Table 2. After washing, the membranes were incubated with peroxidase-coupled secondary antibody for 1 h at room temperature. The blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientist Inc.). We used β-actin as the loading control, in order to normalize our results.

### Remaining glucose in cell supernatant

We considered that low glucose concentrations in cell culture medium corresponded to high glucose uptake by EnSCs. Therefore, we have measured remaining glucose in cell supernatant after 3, 8 and 15 days of differentiation (D3, D8 and D15), using an automated enzyme assay (Roche). Glucose levels were normalized per microgram of total proteins, measured using Bradford method, with BSA as the standard.

### Lactate production

Lactate release into the culture medium by EnSCs was measured using an automated enzyme assay (Roche) after 15 days of cell culture, as previously described (Poidatz et al. 2015, Duval et al. 2016). The level was normalized per microgram of total proteins as described earlier.

### ATP production

EnSCs were seeded onto 12-well culture plates (3.5 × 10^4 cells per well) and lysed on ice in ATP assay buffer. After sonication, cell lysates were centrifuged for 2 min at 15,000g. Cellular ATP production was measured using a dedicated assay kit (ab83355, Abcam), as previously described (Poidatz et al. 2015, Duval et al. 2016). A standard curve is performed for each experiment. According to the fluorometric method, results were analyzed with an Infinite M200 microplate reader at λEX=540 nm/λEM=590 nm (Tecan, San Jose, CA, USA) and normalized per microgram of total proteins as described earlier.

### Glycogen accumulation

EnSCs were seeded in 12-well culture plates (3.5 × 10^4 cells per well). Supernatants were collected in order to measure the extracellular glycogen secretion. In parallel, cells were homogenized in water and boiled 5 min at 100°C to inactivate enzymes. Cell lysates were centrifuged for 5 min at 13,000g. Intracellular and extracellular glycogen accumulation were measured using a dedicated assay kit (MAK016, Sigma) (Salker et al. 2017). A standard curve is performed for each experiment. According to the manufacturer’s instructions, results were analyzed with an Infinite M200 microplate reader at λEX=535 nm/λEM=587 nm (Tecan) and normalized per microgram of total proteins as described earlier.

### Table 1 Primers used for RT-PCR.

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<tr>
<th>Primers</th>
<th>Sequence</th>
<th>PCR product (bp)</th>
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<tr>
<td>GLUT1</td>
<td>5’TGA GCA TCG TGG GCA TCT TT 3’</td>
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<tr>
<td>GLUT3</td>
<td>5’CAG GCA CAC GGT GCA GAT AG 3’</td>
<td>67</td>
</tr>
<tr>
<td>GS</td>
<td>5’AGG CCT TTC CAG AGC ACT TC 3’</td>
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<tr>
<td>GP</td>
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<tr>
<td>TBP</td>
<td>5’TGC TGT CTC CAT GTG TGT ATC T 3’</td>
<td>132</td>
</tr>
<tr>
<td>B2M</td>
<td>5’TGC TGT CTC CAT GTG TGT ATC T 3’</td>
<td>86</td>
</tr>
<tr>
<td>RPL13A</td>
<td>5’CCT GGA GGA GAA GAG GAA GA 3’</td>
<td>125</td>
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### Table 2 Primary antibodies used for immunoblotting.

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<th>Species</th>
<th>Blocking</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Supplier</th>
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<td>1:700</td>
<td>Room temperature</td>
<td>Cell Signaling</td>
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<tr>
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<td>Rabbit</td>
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<td>1:1000</td>
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<tr>
<td>Phospho-GSK3β</td>
<td>Rabbit</td>
<td>BSA 5% (1h)</td>
<td>1:500</td>
<td>+4°C</td>
<td>Cell Signaling</td>
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<tr>
<td>GSK3β</td>
<td>Mouse</td>
<td>BSA 5% (1h)</td>
<td>1:1250</td>
<td>Room temperature</td>
<td>Abcam</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Rabbit</td>
<td>Gelatin 2.5% (2h)</td>
<td>1:500</td>
<td>+4°C</td>
<td>Sigma Aldrich</td>
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<tr>
<td>β-Actin</td>
<td>Rabbit</td>
<td>Milk 5% (1h)</td>
<td>1:1250</td>
<td>Room temperature</td>
<td>Abcam</td>
</tr>
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</table>
VT culture

Human VTs were prepared from tips of placental villi, as previously described (Benaitreau et al. 2010b). Placental villi were incubated five times in HBSS containing trypsin (0.125%), MgSO₄ (4.2 mM), Heps (25 mM), DNase type IV (50 U/mL) at 37°C without agitation. Then, villi were washed several times in warm HBSS. The supernatant containing VTs was collected after tissue sedimentation and centrifuged at 200 g for 10 min. Then, VTs were filtered through 40 µm nylon screen. The cell suspension was layered over a discontinuous Percoll gradient and centrifuged for 25 min at 1000 g. The layer corresponding to 40–45% and 50–55% containing VTs was washed in DMEM/F12 supplemented with FCS (10%), penicillin (10 µg/mL) and streptomycin (100 µg/mL). VTs were seeded in 12-well culture plates (1.5 × 10⁶ cells per well) and cultured in phenol-red-free DMEM/F12 medium supplemented with FCS (10%), streptomycin (10 µg/mL) and penicillin (100 µg/mL) at 37°C under 5% CO₂ and 95% air atmosphere for 24 h. Isolated VTs were characterized by positive staining for cytokeratin-7 (95% stained positive) and the formation of a syncytium. Moreover, cell preparation was not significantly contaminated by the other major placental cell type (extravillous trophoblasts) because the latter require an artificial matrix for growth in vitro (Tarrade et al. 2001). Concomitantly, we studied syncytialization of VTs by measuring hCG secretion. VTs are able to fuse spontaneously after 48–72 h of culture in vitro, as shown the rise in hCG secretion during the cell culture period (Benaitreau et al. 2010b, Poidatz et al. 2014, Duval et al. 2016).

Endometrial glycogen transport from EnSCs to VTs

VTs were seeded in a 12-well culture plate (1.5 × 10⁶ cells per well) and cultured in a phenol-red free DMEM/F12 medium supplemented with streptomycin (10 µg/mL), penicillin (100 U/mL) and FCS (10%) at 37°C in a 5% CO₂ and 95% air atmosphere, for 24 h. Next, VTs were treated with conditioned medium (CM) from 15-day decidualized EnSCs treated or not with human recombinant adiponectin (25 ng/mL or 250 ng/mL). Media from non-decidualized EnSCs were used as controls. After 48 h, glycogen accumulation in placental cells was assayed, as described earlier. The VTs were cultured for a total of 72 h – the time needed to obtain a functional ST (Benaitreau et al. 2010b, Poidatz et al. 2015, Duval et al. 2016).

Flow cytometry analysis of FITC-dextran absorption by VTs

Dextran is a polysaccharide, with a similar structure to glycogen. More precisely, dextran as well as glycogen present α(1,4) and α(1,6) bounds. Thus, it has been suggested that dextran can be used as a glycogen analog for in vitro and in vivo studies (Simionescu et al. 1972, Takikita et al. 2009). In this context, FITC-dextran was used as a fluorescent glycogen analog in this study. VTs were seeded in 12-well culture plates (1.5 × 10⁶ cells per well) and cultured in a phenol-red free DMEM/F12 medium supplemented with streptomycin (10 µg/mL), penicillin (100 U/mL) and FCS (10%) at 37°C in a 5% CO₂ and 95% air atmosphere for 24 h. Next, VTs were cultured in DMEM/F12 medium and FCS (1%) supplemented or not with human recombinant adiponectin (25 ng/mL or 250 ng/mL) for 48 h. Next, cells were stained with FITC-dextran for 2 h at 37°C. Flow cytometry analysis was performed on LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA). Overall, 100,000 events per conditions were acquired based at first on FSC/SSC distribution and secondly on mean FITC fluorescence intensity. First cell gating based on FSC/SSC did remove cell debris but did not allow us to distinct cytotrophoblasts and ST of variable sizes. Cells not exposed to FITC-dextran were used as ‘unstained’ cells and negative control.

Confocal microscopy analysis of fluorescein isothiocyanate (FITC)-dextran absorption by VTs

VTs were seeded in a Labtech culture device (1.0 × 10⁶ cells per well) and cultured in a phenol red-free DMEM/F12 medium supplemented with streptomycin (10 µg/mL), penicillin (100 U/mL) and FCS (10%) at 37°C in a 5% CO₂ and 95% air atmosphere, for 24 h. Next, VTs were cultured in DMEM/F12 medium supplemented with FCS (1%) for 48 h. VTs were exposed to FITC-dextran (Sigma) for 30 min, 1, 2, 3, 4 and 6 h at 37°C. Next, the cells were fixed in paraformaldehyde (4%) for 30 min at room temperature and washed with phosphate buffer saline for 45 min before counterstaining of the cell nuclei with 1 µg/mL Hoechst 33258 Reagent (Sigma). The fluorescence was visualized using an inverted laser scanning confocal microscope (Leica white light laser TCS SP8-X, Leica Microsystems, Wetzlar, Germany). Five cell events per condition were randomly selected and the FITC-dextran intensity (in arbitrary units) was estimated using the post-imaging procedure in ImageJ software. These experiments
were designed to select the most efficient FITC-dextran exposure time for the VTs.

Statistics

Statistical analysis was performed on raw data from 5 to 10 separate experiments and compared with control values (i.e. in the absence of adiponectin) at D3, D8 and D15 in an ANOVA with Bonferroni P value correction. A non-parametric, paired Wilcoxon test was used to assess (i) the effect of the adiponectin concentration (25 ng/mL or 250 ng/mL) with the control situation (the absence of adiponectin) for a given day of exposure.

Results

Effect of adiponectin on glucose transporter and glucose uptake in human EnSCs

We investigated the effect of adiponectin (25 ng/mL or 250 ng/mL) on mRNA expression levels of the glucose transporter isoforms 1 and 3 (GLUT1 and GLUT3) in EnSCs during decidualization. The GLUT1 mRNA expression level increased throughout the decidualization process (4.6-fold higher at D15 of cell culture relative to D3, P<0.01) (Fig. 1A). We found that adiponectin (25 ng/mL) accentuated the increase in GLUT1 mRNA expression (1.54-fold higher at D8 and 1.49-fold higher and D15, P<0.05). Similarly, a significant increased GLUT1 mRNA expression was observed in presence of 250 ng/mL adiponectin (1.94-fold higher at D3, P<0.05; 2.70-fold higher and 2.29-fold higher at D3 and D15, respectively, P<0.01). An opposite expression profile was observed for GLUT3 mRNA expression: in the absence of adiponectin, levels of GLUT3 mRNA expression fell during decidualization (3.1-fold lower at D15 of cell culture relative to D3, P<0.05). Furthermore, neither of the two adiponectin concentrations affected the GLUT3 mRNA expression level at any time point (Fig. 1B). Western blot analysis showed that adiponectin (250 ng/mL) significantly increases GLUT1 expression at D15 of cell culture (3.2-fold higher relative to control) (Fig. 1C). In parallel, we measured the glucose levels in cell supernatants on D3, D8 and D15 of decidualization in the presence or absence of adiponectin (25 ng/mL or 250 ng/mL) (Fig. 1D). Our results showed that adiponectin (25 ng/mL) was associated with significantly lower glucose levels in supernatants (81±7%, P<0.05) at D15 only. However, glucose levels were significantly lower at all time points in the presence of 250 ng/mL adiponectin (91±4, 83±3, and 79±5%, at respectively D3, D8 and D15, P<0.05). Taken as a whole, these results suggest that adiponectin increases glucose uptake by EnSCs.

Effect of adiponectin on glucose catabolism in human EnSCs

In order to determine how decidualized EnSCs use the glucose in the presence of adiponectin, we quantified the cellular ATP levels and lactate release into the cell supernatant. Firstly, we compared cellular ATP levels in undifferentiated (D0) and differentiated (D15) EnSCs. In the absence of adiponectin, we did not observe any significant difference in ATP production between D0 and D15 (67.4±2.8µM and 80.6±12.2µM, respectively)
(data not shown). However, incubation with adiponectin was associated with lower cellular ATP levels on D15 (68.4 ± 10.1% and 65.6 ± 13.5% for 25 ng/mL and 250 ng/mL, respectively, P < 0.05) (Fig. 2A). Secondly, we also observed that the presence of adiponectin was associated with significantly lower lactate production by EnSCs after 15 days of culture (54 ± 12% and 54.4 ± 14.1% for 25 ng/mL and 250 ng/mL, respectively, P < 0.05) (Fig. 2B).

Effect of adiponectin on glycogen synthesis by human EnSCs

As adiponectin did not seem to direct glucose toward anaerobic or aerobic glycolysis, we sought to determine its potential role in glycogen synthesis in human EnSCs. We therefore studied the expression of the main enzymes involved in glycogen hydrolysis (GP) and glycogen synthesis from glucose (GS). In the absence of adiponectin, the GS and GP mRNA expression levels did not change significantly during the EnSC decidualization. However, when EnSCs were treated with adiponectin for 15 days, we observed significantly higher GS mRNA expression (1.71-fold higher and 1.56-fold higher for 25 ng/mL and 250 ng/mL, respectively, P < 0.05) (Fig. 3A). In contrast, we did not observe any effect of adiponectin on GP mRNA expression at either concentration, whatever the time of exposure (Fig. 3B). In parallel, we measured glycogen accumulation in EnSCs on D15 of decidualization. We observed a positive effect of adiponectin on glycogen accumulation for high doses (250, 500 and 1000 ng/mL) (134 ± 10, 184 ± 30 and 139 ± 40%, respectively, P < 0.05). However, in our experimental conditions, we did not observe any significant dose effect of adiponectin (Fig. 3C).

Effect of adiponectin on AMPK and PI3K activation in human EnSCs

Our results suggested that adiponectin promotes glycogen synthesis and conversely blocks glucose catabolism in human EnSCs. Hence, we now sought to specify the signaling pathways involved in these adiponectin effects. Activation of AMPK and PI3K was evaluated by measuring the phosphorylation on the Thr-172 residue of AMPKα and on the Ser-9 residue of GSK3β, respectively. As shown in Fig. 4A and B, we observed that adiponectin-treated EnSCs presented a lower phospho-AMPKα/total-AMPKα ratio at D15 (81.6 ± 4.7% and 74.9 ± 5.5% for 25 ng/mL and 250 ng/mL, respectively, P < 0.05) than non-treated EnSCs. In parallel, a higher phospho-GSK3β/total-GSK3β ratio was observed (169 ± 38% for 250 ng/mL adiponectin, P < 0.05) (Fig. 4C and D).

Signaling pathways involved in adiponectin regulation of glycogen accumulation in human EnSCs

To precise the molecular mechanisms underlying adiponectin effects on glycogen accumulation in human EnSCs, we performed experiments with selective inhibitors and activators of AMPK and PI3K. Compound C (1 µM) and LY294002 (10 µM) were used as AMPK and PI3K inhibitors, respectively. Since AMPK and PI3K are key components of two major transduction pathways in glycogen metabolism, it is not surprising that both inhibitors exerted a significant effect on glycogen accumulation. A significant increase (142 ± 10% relative to the control, P < 0.05) and a significant reduction (68.3 ± 9.7% relative to the control, P < 0.05) in endometrial glycogen accumulation were effectively observed in the presence of Compound C and LY294002, respectively (Fig. 5). We confirmed the importance of PI3K pathway in EnSCs for glycogen accumulation. Indeed, we observed that the increased glycogen accumulation induced by adiponectin persisted in the presence of Compound C (145.6 ± 11.7% relative to the control, P < 0.05) but was abrogated by a treatment with LY294002 (82 ± 11% relative to control, P < 0.05).

Effect of adiponectin on paracrine interaction between EnSCs and VTs

We next sought to specify adiponectin effect on glycogen secretion by EnSCs. To this end, we quantified glycogen secretion into the cell supernatant in the presence or absence of adiponectin (Fig. 6A). Compared with
that EnSCs not exposed to E2 and P4 treatment (i.e. non-decidualized EnSCs) secreted significantly lower amounts of glycogen (39 ± 14% relative to the control, P < 0.05).

In addition, in order to demonstrate whether the endometrial glycogen secreted by EnSCs was captured by placental cells, VT cultures were performed using CM from non-decidualized EnSCs (i.e. EnSCs cultured in the absence of E2 and P4) and from fully decidualized EnSCs treated or not with adiponectin for 15 days (Fig. 6B). First, we observed that VTs cultured with CM from fully decidualized EnSCs, accumulated higher glycogen quantities (+48%) than those cultured with medium from non-decidualized EnSCs. Secondly, we demonstrated that medium enriched in glycogen (i.e. conditioned by adiponectin-treated EnSCs) increased glycogen accumulation in VTs (116 ± 12% and 112 ± 8% for 25 ng/mL and 250 ng/mL, relative to control, P < 0.05).

**Effect of adiponectin on glycogen uptake by human VTs**

As illustrated in Fig. 7A and B, glycogen and dextran molecules are highly similar. Hence, we used FITC-dextran to study glycogen accumulation in VTs. In confocal...
microscopy experiments, we confirmed that (i) VTs accumulated dextran at the concentrations described in the literature (i.e. 1 mg/mL) and (ii) polysaccharide uptake by VTs appears to be optimal after 2 h of VT incubation with FITC-dextran (Fig. 7C). Next, VTs were cultured in the presence of adiponectin (25 ng/mL or 250 ng/mL) for 48 h and then exposed to FITC-dextran (1 mg/mL) for the last 2 h. As shown in Fig. 7D, flow cytometry experiments showed that the mean FITC-dextran fluorescence intensity was significantly lower in VTs exposed to adiponectin (82.4 ± 4.2% and 82.3 ± 8.1% for 25 ng/mL and 250 ng/mL, respectively, relative to control, \( P < 0.05 \)).

**Discussion**

For many years, the endometrium (like the uterus in general) was considered to be an inert organ whose sole function was to support the developing fetus. However, a large body of data clearly now show that the endometrium has a critical role in the establishment of a successful pregnancy. More particularly, EnSCs accumulate and secrete large amounts of nutritive molecules (such as proteins, lipids and glycogen) and provide histiotrophic nutrition to the fetus via endocytosis by the ST (Burton et al. 2007). Endometrial glycogen seems to have a crucial role in the early stages of pregnancy. Indeed, it has been reported that endometrial glycogen levels are lower in infertile women than in fertile women (Maeyama et al. 1977). In order to better understand glycogen metabolism at the fetal-maternal interface, we studied the effects of adiponectin (a key actor of paracrine dialog at the interface) on (i) glucose catabolism and (ii) glycogen accumulation and secretion in human endometrial cells. We also assessed adiponectin effects on glycogen uptake by human trophoblastic cells. In the present study, human recombinant adiponectin had effects at concentrations of 25 ng/mL and 250 ng/mL, which are much lower than those normally found in the main circulation or in cord blood during pregnancy (Kajantie et al. 2004).
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Corbetta et al. 2005). However, the human recombinant adiponectin used in the present study corresponds mainly to the oligomeric form (Bub et al. 2006), which is known to be an active form of adiponectin (Kobayashi et al. 2004). Moreover, adiponectin is commonly used in in vitro experiments with endometrial, placental and other cell types at these sub-physiological concentrations (Brakenhielm et al. 2004, Benaitreau et al. 2009, 2010a, b, Bohlouli et al. 2013, Duval et al. 2016, 2017). We also studied physiological doses of adiponectin (500 ng/mL and 1000 ng/mL) on glycogen accumulation. However, we did not observe any significant dose-dependent effect of adiponectin. This result is in accordance with previous data described in human placenta and endometrium (Benaitreau et al. 2010a, b, Bohlouli et al. 2013, Duval et al. 2016, 2017). In a first set of experiments, we studied glucose catabolism in human EnSCs. In the absence of adiponectin, we observed a major increase over time in mRNA expression of glucose transporter GLUT1 during cell differentiation. Surprisingly, mRNA expression levels of GLUT3 (another glucose transporter isoform expressed in human endometrium) decreased slightly during EnSC differentiation. These results suggest that GLUT1 becomes the major isoform in fully differentiated EnSCs, as previously described in human endometrial and decidual tissues during the menstrual cycle (Strowitzki et al. 2001, Krzeslak et al. 2012).

In parallel, we studied adiponectin effects on GLUT1 and GLUT3 expressions by EnSCs. Our results clearly demonstrated that adiponectin increases GLUT1 expression (mRNA and protein levels) without affecting GLUT3 mRNA expression. These results suggested that adiponectin could promote glucose uptake by increasing specifically GLUT1 expression in EnSCs. Moreover, we showed that exposure to adiponectin was associated with a significant decrease in the catabolic glucose pathways (anaerobic and aerobic glycolysis) and conversely, a relative increase in glucose anabolism (by directly inducing GS mRNA expression, the rate limiting enzyme in glycogen synthesis). These results suggest that adiponectin has a broader range of effects on glucose metabolism in EnSCs than in hepatocytes, a well-characterized cell target in which adiponectin specifically controls glycogenolysis and gluconeogenesis (Combs & Marllis 2014).

Our results also demonstrated that adiponectin favors the accumulation of glycogen in EnSCs. It is well known that the AMPK and PI3K signal transduction pathways are both involved in glycogen metabolism in skeletal muscle and in the liver (Bultot et al. 2012, Khorami et al. 2015). To gain insight into the molecular mechanisms involved in glycogen accumulation in response to adiponectin, we investigated the two main adiponectin pathways. First, our results revealed that adiponectin is able to stimulate PI3K and inversely to inhibit AMPK, suggesting that both ADIPOR1 and ADIPOR2 are functional in EnSCs. Secondly, the results of experiments with specific inhibitors demonstrated that adiponectin promotes glycogen storage specifically via the PI3K transduction pathway. Hence, we can hypothesize that AMPK signaling is involved in other physiological effects of adiponectin such as endometrial glucose catabolism. Further experiments are underway in our laboratory.

It is well established that glycogen secretion by endometrial cells is an apocrine secretory mechanism (Bergeron 2000, Demir et al. 2002). More precisely, intracellular glycogen-enriched vacuoles appear in endometrial cell cytoplasm, under the influence of progesterone. These vacuoles induce the detachment
of the apical region of endometrial cells resulting in the glycogen discharge from the endometrial cell. In this context, we have studied the direct effects of adiponectin on glycogen secretion by EnSCs during in vitro decidualization. We found that glycogen secretion appears to depend on the EnSC decidualization status since EnSCs cultured in the absence of E2, and P4 secreted less glycogen than fully decidualized EnSCs. These data confirm and complete several previous studies performed with endometrial glands demonstrating that glycogen content is associated with decidualization status (Salameh et al. 2006, Rose et al. 2011, Dean et al. 2014, Filant & Spencer 2014). Moreover, we showed that EnSCs treated with adiponectin secreted significantly higher amounts of glycogen than non-treated cells. Taken as a whole, our results clearly demonstrated that adiponectin improves endometrial glycogen secretion by stimulating the endometrial glycogen accumulation.

We next sought to establish whether placental cells are able to take up endometrial glycogen. To this end, VTs were plated in the presence of CM from EnSCs cultured in the presence or in the absence of adiponectin. We clearly demonstrated that VTs treated with glycogen-enriched medium (i.e. CM from adiponectin-treated EnSCs) accumulate greater amounts of glycogen in their cytoplasm than VTs treated with low-glycogen medium (i.e. CM from EnSCs not treated with adiponectin or EnSCs not treated with E2 and P4). These results suggest that VT glycogen uptake (i) depends on the amount of glycogen in the extracellular medium and (ii) seems to be controlled by adiponectin. In fact, our experiments performed with FITC-dextran revealed that adiponectin directly controls trophoblast glycogen uptake. Adiponectin can also indirectly control this process by stimulating the secretion of endometrial factors. Epidermal growth factor seems to be a good candidate since it is secreted by EnSCs and is known to increase glycogen uptake by hepatocytes (Freemark 1986, Burton et al. 2007).

Lastly, we found that internalized glycogen co-localized first with endosomes (Supplementary Fig. 1, see section on supplementary data given at the end of this article) and then with lysosomes (Supplementary Fig. 2). The latter results are in line with the literature data on the trafficking of macromolecules (including glycogen) between decidual glands and trophoblasts (Jones et al. 2015). Hence, we can hypothesize that after placental endocytosis, endometrial glycogen is degraded (like proteins) in lysosomes (Supplementary Figs 1, 2 and 3). The newly formed glucose molecules are probably used by VTs for their own metabolic needs or transmitted to the fetus via the histiotrophic nutrition pathway. Further experiments will be needed to confirm this hypothesis and to specify adiponectin roles in placental glycophagy.

In conclusion, our present results show that adiponectin promotes glycogen synthesis, accumulation and secretion by human EnSCs. However, adiponectin also slows down glycogen entry into human VTs, suggesting that adiponectin downregulates the placenta uptake of

Figure 8
Proposed mechanism by which adiponectin controls glycogen metabolism and trafficking at the fetal–maternal interface. In human EnSCs, adiponectin stimulates glucose uptake by upregulating GLUT1 mRNA expression. Then, adiponectin significantly reduces the catabolic glucose pathways (anaerobic and aerobic glycolysis) and conversely increases both glycogen synthesis (by directly inducing GS mRNA expression) and glycogen accumulation. This last effect is, in part, mediated by PI3K transduction signaling. Glycogen secretion being an apocrine mechanism, adiponectin also improves glycogen secretion. At the fetot–maternal interface, adiponectin limits glycogen uptake by VTs. Once endocytosis, glycogen is directed toward lysosomes in order to be degraded into glucose. Glucose could then be used by placental cells for its own metabolic needs, or transmitted to the fetus in order to insure fetal nutrition. GS, glycogen synthase; ↗, increase; ↘, inhibition. A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0013
nutrients through the histiotrophic nutrition pathway. Indeed, we and others have reported that adiponectin inhibits nutrient transporters expression in the first-trimester and third-trimester human placenta as well as in the murine placenta ([Caminos et al. 2005, Jones et al. 2010, Aye et al. 2014, Duval et al. 2016]). Interestingly, opposite effects of adiponectin in the endometrium and the placenta have been already described. Indeed, we found that adiponectin induces pro-invasive effects in human VTs ([Benaitreau et al. 2010b]) but anti-invasive effects in human EnSCs ([Duval et al. 2017]). Furthermore, we demonstrated that adiponectin promotes aerobic glycolysis and ATP synthesis in human placental cells ([Duval et al. 2016]). In the present study, we found that adiponectin had the opposite effect in human EnSCs. Moreover, adiponectin has proinflammatory effects in the endometrium and anti-inflammatory effects in the placenta ([Lappas et al. 2005, Takemura et al. 2006]).

Studies performed in our laboratory revealed that adiponectin exerts pro-differentiative, pro-invasive and pro-apoptotic effects in human placental cells and conversely, anti-differentiative and anti-invasive effects in human EnSCs. Our work highlighted a novel effect of adiponectin in both cell types. Taken as a whole, our present data suggest that this adipokine is a major actor at the fetal–maternal interface. Thus, dysfunctional adiponectin signaling (due to a low endometrial ADIPOR expression, for example) might jeopardize a successful pregnancy – as we have shown previously in our study of endometrial tissue from women with unexplained recurrent implantation failure ([Santos et al. 2012]). The role of adiponectin in glycogen metabolism and trafficking at the fetal–maternal interface during the first trimester of pregnancy is summarized in Fig. 8.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0013.

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

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