Adiponectin limits differentiation and trophoblast invasion in human endometrial cells

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Abstract

Successful human embryo implantation requires a proper differentiation of endometrial stromal cells (ESCs) into decidual cells, during a process called decidualization. ESCs express specific molecules, such as prolactin, insulin-like growth factor-binding protein-1 (IGFBP-1) and connexin-43. Decidual cells are also involved in the control of trophoblast invasion, by secreting various factors, such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Adiponectin is an adipokine with insulin-sensitizing, anti-inflammatory and anti-proliferative effects. At the embryo–maternal interface, adiponectin promotes differentiation and invasion of human trophoblastic cells. We hypothesize that the effects of adiponectin on endometrium could counteract its pro-invasive effects previously described in the human trophoblast. In this context, we have firstly demonstrated that adiponectin downregulates IGFBP-1 and connexin-43 mRNA expressions, as well as prolactin secretion in ESCs, suggesting an anti-differentiative effect of adiponectin. Secondly, we found that invasive capacities of trophoblastic cell line HTR-8/SVneo are reduced in the presence of conditioned media from ESC cultured in the presence of adiponectin. Adiponectin's anti-invasive action is associated with a decreased activity of MMP-2 and MMP-9, and an increased TIMP-3 mRNA expression in ESCs. Finally, adiponectin receptors (*ADIPOR1* and *ADIPOR2*) knockdown abolishes the anti-differentiative and anti-invasive effects of adiponectin in human ESCs. Altogether, our results suggest that adiponectin reduces the decidualization process and inversely induces the production of endometrial factors that limit trophoblast invasion. Thus, through a dual control in trophoblast and endometrial cells, adiponectin appears as a pivotal actor of the embryo implantation process.

Key Words

- ▶ adiponectin
- ▶ decidualization
- ▶ embryo implantation
- ▶ human endometrium
- ▶ trophoblast invasion

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Introduction

Human embryo implantation is a multi-step process that begins with apposition of trophoblastic cells from a competent blastocyst to the maternal endometrium. This

apposition occurs during the 'window of implantation', a short period of uterine receptivity corresponding to the mid-secretory phase of the menstrual cycle

(Diedrich *et al.* 2007). After the blastocyst has attached to the endometrial epithelium, its extravillous trophoblasts (EVTs) penetrate into the endometrial stroma and acquire an invasive phenotype resulting in the placenta anchoring in the endometrium (Lunghi *et al.* 2007).

During trophoblast invasion, EVT's secrete large amounts of matrix metalloproteinases (MMPs) (Burrows *et al.* 1996). The actions of MMP-2 and MMP-9 are crucial for trophoblast invasion (Staun-Ram *et al.* 2004). Indeed, these gelatinases are able to degrade the major component of the endometrial extracellular matrix (ECM) i.e. collagen IV (Burrows *et al.* 1996). MMP activity is regulated by specific tissue inhibitors of metalloproteinases (TIMPs), which bind and inactivate MMPs with a 1:1 stoichiometry (Huppertz *et al.* 1998). Thus, correct regulation of the MMP/TIMP balance in EVT's is essential for an appropriate embryo implantation (Nissi *et al.* 2013). Trophoblast invasion also requires adequate endometrial receptivity (Singh *et al.* 2011). This is characterized by morphological, biochemical and vascular modifications, including (i) the appearance of epithelial pinopodes, (ii) the modulated expression of cytokines, growth factors and adhesion molecules (particularly the upregulation of epithelial α v- and β 3-integrins), (iii) the disappearance of an adhesive molecular surface (loss of mucin-1 and mucin-12) and (iv) the decidualization of endometrial stromal cells (ESCs). Decidualization occurs in response to the ovarian hormones, 17 β -estradiol (E2) and progesterone (P4). It is accompanied by the secretion of prolactin and insulin-like growth factor-binding protein-1 (IGFBP-1), and the expression of the gap junction connexin-43 (CX-43) in human ESCs (Brar *et al.* 1995, Yu *et al.* 2011). Decidual ECM remodeling events have also been observed. These processes are associated with MMP-2,-9 secretion and the modulation of *TIMP-1,-2,-3* expression in human ESCs (Zhang & Salmonsens 1997, Estella *et al.* 2012, Tapia-Pizarro *et al.* 2013). This type of ECM remodeling enhances ESC motility and thus facilitates trophoblastic infiltration into the endometrial stroma (Grewal *et al.* 2008). Decidual ECM remodeling is a complex process, tightly controlled by factors intrinsically produced by both placental and decidual cells (Sharma *et al.* 2016). For example, human chorionic gonadotropin (hCG) and tumor necrosis factor alpha (TNF- α) facilitate EVT migration by enhancing endometrial MMP-2,-9 activities and by inhibiting endometrial *TIMP-1* secretion (Haider & Knöfler 2009, Tapia-Pizarro *et al.* 2013). Conversely, the antifungal antibiotic trichostatin A limits trophoblast invasion by increasing the production of *TIMP-1,-3* by

ESCs and reducing the activity of endometrial MMP-2,-9 (Estella *et al.* 2012). Hence, the local MMP/TIMP balance, at the invasion site, requires an appropriate interaction between trophoblasts and ESCs.

Adiponectin is a 30kDa cytokine, predominantly produced by adipose tissue (Kern *et al.* 2003). Adiponectin exerts its action *via* two specific receptors (ADIPOR1 and ADIPOR2) containing seven transmembrane domains but which are structurally and functionally distinct from G-protein-coupled receptors (Yamauchi *et al.* 2003a). By binding to ADIPORs, adiponectin activates a variety of targets, such as protein kinase A, phosphoinositide-3 kinase, mitogen-activated protein kinase and most notably, the AMP-activated protein kinase through which adiponectin exerts its insulin-sensitizing effects (Yamauchi *et al.* 2003a, Campos *et al.* 2008). This adipokine has also been shown to exert anti-inflammatory, anti-angiogenic, anti-atherosclerotic, anti-proliferative and pro-apoptotic roles in various cell types (Yamauchi *et al.* 2003b, Dieudonne *et al.* 2006, Duval *et al.* 2016).

During pregnancy, adiponectin seems to act as an endo/paracrine signal at the embryo-maternal interface. It is now established that adiponectin is not produced by human trophoblastic cells but seems to be expressed in human endometrium (Takemura *et al.* 2006, Benoitreau *et al.* 2010a, Dos Santos *et al.* 2012). ADIPOR1 and ADIPOR2 are expressed in human placenta and endometrium (Takemura *et al.* 2006, Benoitreau *et al.* 2009). A growing body of evidence has highlighted adiponectin role in the establishment of a functional placenta (Benoitreau *et al.* 2010b). Our laboratory has shown that adiponectin promotes human trophoblast invasion by enhancing MMP-2,-9 activities and downregulating *TIMP-2* mRNA expression (Benoitreau *et al.* 2010a).

However, little is known about adiponectin effects in human endometrium. A study showed that expression of ADIPOR1 and ADIPOR2 peaks in the mid-secretory phase of menstrual cycle (Takemura *et al.* 2006). We have also observed significantly low endometrial expression of ADIPOR1 and ADIPOR2 in women with unexplained recurrent implantation failure relative to fertile women (Dos Santos *et al.* 2012). *In vitro*, adiponectin exerts an anti-proliferative action by promoting cell death (Bohlouli *et al.* 2013). Taken as a whole, these data strongly suggest that adiponectin plays a crucial role during embryo implantation. With a view to better understand embryo-maternal dialog in the early stages of pregnancy, we studied the *in vitro* effects of adiponectin on (i) the human ESC decidualization program and (ii) the endometrial control of trophoblast invasion.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium and Ham F-12 Nutrient Mix (DMEM/F12), Roswell Park Memorial Institute (RPMI) medium, progesterone, E2, penicillin, streptomycin, DNase type I, EDTA, leupeptin, aprotinin, 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), sodium fluoride 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). Nucleospin RNA II kit was obtained from Machery-Nagel (Düren, Germany). Trypsin was provided by Difco Laboratories (Detroit, MI, USA). Matrigel was obtained from BD Biosciences (Le Pont-de-Claix, France) and collagenase A was from Boehringer (Mannheim, Germany). The suppliers of the various antibodies used are described in the corresponding paragraphs below.

Study population and tissue collection

A total of 23 normally cycling women aged 27–40 undergoing biopsy for fertility evaluation, were recruited into the present study. Endometrial tissues were obtained during the window of implantation. Patients with low endometrial volume (<2 cm) or presence of submucous fibroma or polyps were excluded. A good hormonal reserve and normal responses to ovarian stimulation were also included as additional criteria. This 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 informed consent before tissue sampling.

Human ESC culture

Human ESCs were isolated and cultured as described by González and coworkers (Gonzalez *et al.* 1999). Briefly, tissue samples were minced into small pieces and digested in a two-step process. Tissues were incubated for 1 h at 37°C in a phenol-red-free DMEM/F12 medium containing collagenase (0.1%), DNase type I (0.0005%), penicillin (10 µg/mL) and streptomycin (100 U/mL).

The supernatant was filtered through a 100 µm nylon screen and then centrifuged at 200g for 10 min. A second enzymatic digestion was performed on undigested tissue for 10 min at 37°C in DMEM/F12 medium-containing trypsin (0.25%), DNase type I (0.1%), EDTA (0.03%), penicillin (10 µg/mL) and streptomycin (100 U/mL). The digested tissue was filtered through a 40 µm nylon screen and then centrifuged at 200g for 10 min. Cell pellets from both digestions were pooled and centrifuged at 200g for 10 min.

Cells were cultured in 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. When the cells reached confluence, they were cultured in the presence or absence of adiponectin (25 ng/mL and 250 ng/mL) in differentiation medium containing DMEM/F12 with E2 (10⁻⁸ M), P4 (10⁻⁶ M), charcoal-stripped FCS (2%), penicillin (10 µg/mL) and streptomycin (100 U/mL) for 15 days. Media were changed every 2 days.

Immunocytochemistry

Endometrial cells were washed three times in phosphate-buffered saline (PBS) and fixed with paraformaldehyde (4%) for 30 min at room temperature. Cells were permeabilized for 5 min with PBS-Triton X-100 (0.2%). Non-specific binding was prevented by incubation with BSA (3%) in PBS for 1 h at room temperature. Vimentin (mouse anti-human monoclonal, 1:50, clone V9, 790-2917, Ventana Medical Systems, Tucson, AZ, USA) and pan-cytokeratin (mouse anti-human monoclonal, 1:50, clone KL1, Mob 191-05, Diagnostic Biosystems, Pleasanton, CA, USA) were chosen as stromal and epithelial markers, respectively (Merviel *et al.* 1995). The labeled cells were then rinsed with PBS and incubated with an Alexa-488-conjugated goat-anti-mouse secondary antibody (1:400, A_{max}/E_{max}: 488/519, A-11001, Molecular Probes, Thermo Fisher Scientific Inc.) for 1 h at room temperature. Cell nuclei were counterstained with 1 µg/mL Hoechst 33258 (Sigma-Aldrich). As a negative control, endometrial cells were processed in the absence of primary antibody. Immunostained samples were observed using an automated inverted microscope (ScanR, Olympus, Rungis, France). Positive cells were counted on five randomly selected fields by the post-imaging procedure in ImageJ software.

Invasion assay for HTR-8/SVneo cells co-cultured with ESC culture medium

The invasiveness of trophoblastic cells in response to ESC-secreted molecules was assessed using the HTR-8/SVneo immortalized EVT cell line, kindly provided by Dr Nadia Alfaidy (CEA Grenoble, France) in agreement with Dr Charles Graham. HTR-8/SVneo cells were cultured in RPMI medium supplemented with HEPES 1M (2%), penicillin (100 U/mL), streptomycin (100 µg/mL) and FCS (10%) until they reached confluence. Invasion assays were performed in 24-well plates containing Matrigel-coated polycarbonate membrane (pore size: 8 µm) invasion chamber inserts (Greiner Bio-One SAS, Courtaboeuf, France), according to the modified protocol from Tapia-Pizarro and coworkers (Tapia-Pizarro *et al.* 2013). HTR-8/SVneo cells were suspended (5×10^4 cells per well) in 250 µL of conditioned medium (CM) from 15-day-decidualized ESCs treated or not with adiponectin (25 ng/mL and 250 ng/mL). Media containing only E2 and P4 in the absence or presence of adiponectin (25 ng/mL and 250 ng/mL), were used as controls. RPMI medium supplemented with FCS (10%) was added to the lower well as a chemoattractant. After 48 h of incubation at 37°C, medium containing non-invading cells was removed from the upper well. Invasive HTR-8/SVneo cells at the lower surface of the insert were washed and fixed with paraformaldehyde (4%) for 30 min. The nuclei were counterstained with 1 µg/mL Hoechst reagent and visualized with an inverted laser scanning confocal microscope (Leica white light laser TCS SP8-X, Leica Microsystems, Wetzlar, Germany). On each insert, invasive cells were counted on five randomly selected fields by the post-imaging procedure in ImageJ software. Invasive cells were defined as those whose nucleus exceeded 8 µm (i.e. the equivalent of a pore size).

Zymography

After 1, 8 or 15 days of culture (D1, D8 and D15) with differentiation medium in the absence or presence of adiponectin (25 ng/mL and 250 ng/mL), total gelatinase activities from ESCs were analyzed by zymography. Aliquots of CM containing 60 µg of protein were resolved under non-reducing conditions in 10% polyacrylamide gels containing 1 mg/mL gelatin (Difco). Gels were washed in Triton X-100 (2.5%) for 30 min to remove SDS and incubated overnight at 37°C in a renaturing buffer (50 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 150 mM NaCl, and 0.02% sodium azide). Gels were stained with Coomassie Brilliant Blue, and destained in methanol/acetic acid

(20%/5% v/v). Proteolytic activity was identified as a clear band on a blue background. The images were scanned and quantitative enzyme analysis was carried out, using ImageJ software.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

ESCs were seeded in 12-well culture plates (3.5×10^5 cells per well). After 3, 8 or 15 days of differentiation (D3, D8, D15), total RNA (0.1 µg) from ESCs was extracted and reverse-transcribed, as previously described (Machinal-Quélin *et al.* 2002). Quantitative PCR was performed using the C1000 Thermal Cycler (CFX96 real-time system; BioRad, Hercules, CA, USA) and the primers sets indicated in Table 1. Final concentration of primers is 25 µM. The three reference genes, ribosomal protein L13A (RP13), TATA box-binding protein (TBP) and β2-microglobulin were chosen as previously described (Benaitreau *et al.* 2009). For each sample, the concentration ratios (target/three reference mRNA) were calculated using the BioRad CFX Manager software (version 3.0) and expressed in arbitrary units, as previously described (Duval *et al.* 2016).

PCR products were separated on a 2% agarose gel in 90 mM Tris-borate and 2 mM EDTA buffer (pH 8.0). They were visualized by staining with GelRed (Interchim, Montluçon, France) and UV transillumination.

Immunoblotting

ESCs were seeded onto 6-well culture plates (2.0×10^6 cells per 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 total proteins were resolved by SDS-PAGE (4–20% acrylamide) and wet-transferred onto nitrocellulose membranes. Next, the membranes were blocked and incubated overnight with anti-ADIPOR1 (goat anti-human polyclonal, 1:300, sc-46749, Santa Cruz Biotechnology), anti-ADIPOR2 (goat anti-human polyclonal, 1:300, sc-46756, Santa Cruz Biotechnology) or anti-β-actin (rabbit anti-human monoclonal, 1:500, Sigma Chemical). After washing, the membranes were incubated with the peroxidase-coupled secondary antibody, for 1 h at room temperature. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Data were analyzed using ImageJ software.

Table 1 Primers used for RT-PCR.

Primer set	Sequence	PCR product	Sequence
ADIPOR1			
Sense	5'TTC TTC CTC ATG GCT GTG ATG T 3'	71 bp	NM_001290629.1
Antisense	3'GCT TAA GGA CTC GCG AAG AA 5'		
ADIPOR2			
Sense	5'ATA GGG CAG ATA GGC TGG TTG A 3'	76 bp	NM_024551.2
Antisense	3'ACA TAC GAC GGG CCT AGG 5'		
ADIPONECTIN			
Sense	5'GTC ATT GTC ATT ATC AGC 3'	153 bp	NM_004797.3
Antisense	3'TAT CCA CTT CTC GTA TCG 5'		
PROLACTIN			
Sense	5'AGC CAG GTT CAT CCT GAA A 3'	99 bp	NM_000948.5
Antisense	3'TTC TCA GAG CGG AAA GAC GA 5'		
IGFBP-1			
Sense	5'ATC ACA GCA GAC AGT GTG AGA 3'	71 bp	NM_000596.2
Antisense	3'CCA CGC AGA TGG GAA CCT TA 5'		
CONNEXIN-43			
Sense	5'TTA AGC AAA AGA GTG GTG CCC 3'	179 bp	NM_000165.4
Antisense	3'GAC CCC TCT ACT CGT CAG AC 5'		
TIMP-3			
Sense	5'CAC CTC TCC ACG AAG TTG C 3'	117 bp	NM_000362.4
Antisense	3'GTG TCT CTG AGA GCC TTC GA 5'		
TBP			
Sense	5'TGC ACA GGA GCC AAG AGT GAA 3'	132 bp	NM_001172085.1
Antisense	3'CAC ATC ACA GCT CCC CAC CA 5'		
B2-MICROGLOBULIN			
Sense	5'TGC TGT CTC CAT GTT TGA TGT ATC T 3'	86 bp	NM_004048.2
Antisense	3'TCT CTG CTC CCC ACC TCT AAG T 5'		
RPL13A			
Sense	5'CCT GGA GGA GAA GAG GAA AGA GA 3'	125 bp	NM_012423.3
Antisense	3'TTG AGG ACC TCT GTG TAT TTG TCA A 5'		

RNA interference for ADIPOR1 and ADIPOR2

Two pairs of small-interfering RNAs (siRNAs) corresponding to different regions of each receptor gene were chemically synthesized by Qiagen. The sequences of the sense siRNAs are given in Table 2. A fluorescently labeled, non-silencing control siRNA (siNS) was used to optimize the transfection conditions and as a control for non-specific silencing effects. For the knockdown experiments, ESCs were plated in 12-well dishes at 3.5×10^5 cells per well. After 13 days, cells were transfected with siNS (10nM) or siADIPOR1 (25nM) or siADIPOR2 (25nM) using a Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. Adiponectin (250ng/mL) was added 24h after

transfection. mRNA expression was analyzed by RT-qPCR and protein expression was assessed using Western blot, as described previously.

Prolactin secretion

Prolactin secretion into the culture medium was measured using an automated immuno-chemiluminescence system (KRYPTOR, B·R·A·H·M·S, Saint-Ouen, France). In order to compare the levels of prolactin secretion, results were normalized to 1µg of total protein. The protein concentration was measured according to Bradford's method, with BSA as the standard.

Statistical analyses

Statistical analyses were performed on the raw data from 5 to 10 separate experiments. Comparisons between controls (absence of the adiponectin) at D1, D3, D8 and D15, were made using analysis of variance (ANOVA) with Bonferroni *P* values. A non-parametric, paired Wilcoxon test was used to compare the effect of adiponectin concentration

Table 2 siRNA sequences.

Target	Sequence
ADIPOR1	5'AAG GAC AAC GAC TAT CTG CTA 3' 5'CTG GCT AAA GGA CAA CGA CTA 3'
ADIPOR2	5'ACC AAT TTA AGT GAA CAT TTA 3' 5'CGG CTC TCC TTG AAT AAG AAA 3'

Figure 1

Isolated endometrial cells are human ESCs. After isolation, human endometrial cells were cultured in DMEM/F12 medium supplemented with E2 and P4. Vimentin (a stromal marker) and pan-cytokeratin (an epithelial marker) were revealed as described in the Materials and methods section. (A) Human endometrial cell stained with anti-vimentin antibody. (B) Human endometrial cell stained with anti-pan-cytokeratin antibody. (C) Negative staining control in the absence of primary antibody. The figure shows one of four separate, representative experiments. Magnification $\times 40$.

(25 ng/mL or 250 ng/mL) with the control situation (the absence of adiponectin) for a given day of exposure.

Results

Characterization of human ESCs

To assess the purity of our cell preparation, immunocytochemistry experiments were performed, 48 h after cell isolation. We have studied the expression of vimentin and pan-cytokeratin, which are specific markers of endometrial stroma and epithelium, respectively. Our microscopy analyses revealed that 95% of isolated endometrial cells stained positive for vimentin and only 5% expressed pan-cytokeratin (Fig. 1). This clearly demonstrated that the great majority of isolated cells were ESCs. These latter results were confirmed by measuring prolactin secretion into cell culture supernatant, which is a specific marker of ESC decidualization. We showed

that this hormonal production increased progressively during cell differentiation (16 ± 3 , 66 ± 16 , 571 ± 157 , and 2374 ± 607 IU/ μ g of protein after 1, 3, 8, and 15 days of cell culture, respectively).

Expression of adiponectin and its specific receptors in human ESCs

Human ESCs did not express adiponectin at D3, D8 and D15 of cell decidualization. However, this adipokine was expressed in human adipose tissue, used as a positive control (Fig. 2A). The RT-qPCR results demonstrated that ESCs expressed *ADIPOR1* and *ADIPOR2*, whatever the decidualization status. Furthermore, the respective expression levels were 2.1- and 2.3-fold higher at D15 of cell culture, over D3 (Fig. 2B). Western blot analyses confirmed the presence of the two ADIPOR subtypes in human ESCs, at D3, D8 and D15 of cell decidualization. Human first-trimester placenta was used as a positive control (Fig. 2C).

Figure 2

Adiponectin receptors are expressed in human ESCs. Human ESCs were cultured in DMEM/F12 medium supplemented with E2 and P4. (A) Total RNA was extracted after 3 days (D3), 8 days (D8) and 15 days (D15) of cell differentiation. Adiponectin, ADIPOR1 and ADIPOR2 mRNA expression levels were analyzed by RT-PCR with the primers listed in Table 1. PCR products were analyzed by agarose gel electrophoresis. The figure represents one representative of nine separate experiments. (B) ADIPOR1 and ADIPOR2 mRNA levels were quantified by RT-qPCR as described in Materials and methods section. The data are quoted as the mean $\pm$ S.E.M. of nine separate experiments. (C) Western blot analysis of cell lysates (20 μ g) using anti-ADIPOR1 and anti-ADIPOR2 antibodies, as described in the Materials and methods section. The figure shows one representative of five separate experiments. * $P < 0.05$; ** $P < 0.01$; NS: non-significant. ANOVA test.

However, ESC treatment with adiponectin (25 ng/mL and 250 ng/mL) did not seem to affect *ADIPOR* expression in these cells (data not shown).

Effect of adiponectin on human ESC decidualization

Prolactin, *IGFBP-1* and *CX-43* mRNA expressions and prolactin secretion were used as biochemical markers in order to study the differentiation of ESC into decidual cells. As shown in Fig. 3A, B and C, we observed all along the ESC decidualization process, a significant increasing prolactin, *IGFBP-1* and *CX-43* mRNA expressions, with a maximal expression at D15 (8.8-fold, 715.2-fold and 3.3-fold increase over D3, respectively). Likewise, we observed a rise in prolactin secretion between D1 and D15 of cell decidualization

(118.4-fold) (Fig. 3D). These results validated our *in vitro* differentiation protocol and showed that ESCs are fully decidualized at D15.

Next, we studied adiponectin effect on decidualization. We did not observe an effect of adiponectin on prolactin mRNA expression, at D3, D8 and D15, for both doses tested (Fig. 3A). However, we showed a drastic decrease in prolactin secretion at D8, in the presence of 250 ng/mL adiponectin (–25%) and at D15, for the two doses tested (–27% at 25 ng/mL and –49% at 250 ng/mL) (Fig. 3D). In presence of adiponectin, we also observed a significant decrease in *IGFBP-1* mRNA expression (–72% at 25 ng/mL and –53% at 250 ng/mL) and *CX-43* mRNA expression (–41% at 25 ng/mL and –37% at 250 ng/mL) at D15 of cell culture only (Fig. 3B and C).

Figure 3

Adiponectin limits ESC decidualization. Human ESCs were cultured in DMEM/F12 medium supplemented with E2 and P4, and exposed or not to adiponectin (25 ng/mL and 250 ng/mL) for 15 days. (A, B and C) Total RNA was extracted after 3 days (D3), 8 days (D8) and 15 days (D15) of cell differentiation. Prolactin, *IGFBP-1* and connexin-43 mRNA levels were quantified by RT-qPCR, as described in the Materials and methods section. The data are quoted as the mean $\pm$ s.e.m. of nine separate experiments. (A) Prolactin mRNA levels. (B) *IGFBP-1* mRNA levels. (C) Connexin-43 mRNA levels. (D) Prolactin secretion into the endometrial supernatants was measured after 1 day (D1), 3 days (D3), 8 days (D8) and 15 days (D15) of cell differentiation, as described in the Materials and methods section. The data are quoted as the mean $\pm$ s.e.m. of ten separate experiments. The control values were 16.75 ± 3.07 , 66.34 ± 16.67 , 571.35 ± 157.07 and 2374.68 ± 607.50 IU/g proteins for D1, D3, D8 and D15, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: non-significant. (A) vs control situation (without adiponectin). Wilcoxon test. (B) vs D3; (C) vs D8; (D) vs D1. ANOVA test.

Involvement of adiponectin in the endometrial control of trophoblast invasion

In order to determine how adiponectin might be involved in the endometrial control of trophoblast invasion, we performed Matrigel Transwell invasion assays. We cultured the HTR-8/SVneo immortalized EVT cell line in the presence of CM collected from differentiating ESCs treated or not with adiponectin (25 ng/mL and 250 ng/mL) for 15 days (Fig. 4A). As shown in Fig. 4B, we observed that CM from ESCs treated with adiponectin significantly reduced the HTR-8/SVneo cell invasive capacities (−17% at 25 ng/mL and −32% at 250 ng/mL). In parallel, we performed Matrigel Transwell invasion assays with the HTR-8/SVneo cells in the presence of control medium supplemented or not with adiponectin (25 ng/mL and 250 ng/mL). As previously described by our laboratory, these experiments confirmed that adiponectin (250 ng/mL) enhanced trophoblast invasion (+23%) (Benaitreau *et al.* 2010a).

Figure 4

Adiponectin improves the endometrial control of trophoblastic migration. (A) Transwell migration assays of HTR-8/SVneo cells were performed as described in the Materials and methods section. (B) HTR-8/SVneo cells were suspended in the presence of conditioned medium (CM) from 15-day decidualized ESCs treated or not with adiponectin (25 ng/mL and 250 ng/mL) or in the presence of control medium, supplemented or not with adiponectin (25 ng/mL and 250 ng/mL). The data are quoted as the mean $\pm$ S.E.M. of seven separate experiments. * $P < 0.05$; ** $P < 0.01$; NS: non-significant. Wilcoxon test.

Effect of adiponectin on endometrial TIMP mRNA expression

We next sought to specify the molecular mechanisms involved in adiponectin anti-invasive action. We focused on the mRNA expression of three invasion inhibitors, such as *TIMP-1*, *TIMP-2*, and *TIMP-3*, strongly expressed in the human endometrium. Using RT-qPCR, we found that *TIMP-1* and *TIMP-2* mRNA expression did not significantly change throughout ESC decidualization regardless of the presence or absence of adiponectin (data not shown). However, as shown in Fig. 5A, *TIMP-3* mRNA expression increased significantly throughout the ESC decidualization process with a peak effect at D15 of cell culture (1.8-fold increase over D3). Furthermore, this increase was amplified by the presence of 25 ng/mL adiponectin (+117%, +81%, +69%, on D3, D8 and D15, respectively) and 250 ng/mL adiponectin (+122%, +100% and +125% at D3, D8 and D15, respectively).

Effect of adiponectin on endometrial MMP activity

Zymography assays revealed two bands corresponding to the gelatinase activities of MMP-2 (72 kDa) and MMP-9 (92 kDa) in CM collected on D1, D8 and D15 of cell culture. Both gelatinases showed a higher activity at D8 and D15. An additional band of gelatinase activity (45 kDa) appeared in CM collected at D1 but had disappeared by D8 and D15 of cell culture (Fig. 5B). Figure 5C and D showed that adiponectin significantly decreased MMP-2 and MMP-9 activities at D8 (−19% for both MMPs) but only at the higher dose tested. At D15, however, we found that adiponectin downregulated MMP-2 activity (−11% at 25 ng/mL and −15% at 250 ng/mL) and MMP-9 activity (−28% at 25 ng/mL and −31% at 250 ng/mL).

Effects of ADIPOR1 and ADIPOR2 knockdown in human ESCs

Considering the late effect of adiponectin on decidualization (significant at D15 of differentiation), we decided to knockdown *ADIPOR1* and *ADIPOR2* mRNA expressions with siRNA for the last 2 days of decidualization (from D13 to D15). We observed a significant decrease in *ADIPOR1* and *ADIPOR2* mRNA expression levels (−56 and −58%, respectively) after 48 h of knockdown. This ADIPOR invalidation was confirmed using Western Blot analysis (Fig. 6).

Figure 5

Adiponectin modifies endometrial TIMP expression and MMP activities. Human ESCs were cultured in DMEM/F12 medium supplemented with E2 and P4, and exposed or not to adiponectin (25 ng/mL and 250 ng/mL) for 15 days. (A) Total RNA was extracted after 3 days (D3), 8 days (D8) and 15 days (D15) of cell differentiation. The data are quoted as the mean $\pm$ s.e.m. of ten separate experiments. (B) Activities of gelatinases in conditioned media (CM) from decidualized ESCs after 1 day (D1), 8 days (D8) and 15 days (D15) of cell differentiation were measured as described in the Materials and methods section. This figure shows one representative of seven separate experiments. (C) Quantification of the gelatin zymography results for the MMP-2 band. The data are quoted as the mean $\pm$ s.e.m. of seven separate experiments. (D) Quantification of the gelatin zymography results for the MMP-9 band. The data are quoted as the mean $\pm$ s.e.m. of seven separate experiments. * $P < 0.05$; ** $P < 0.01$; NS: non-significant. (A) vs control situation (without adiponectin). Wilcoxon test. (B) vs D3; (C) vs D8. ANOVA test.

The partial suppression of *ADIPOR1* and *ADIPOR2* abolished the decrease of *CX-43* and *IGFBP-1* mRNA expressions observed in the presence of 250 ng/mL adiponectin (Fig. 7). In parallel, silencing the *ADIPOR1* or *ADIPOR2* genes suppressed adiponectin (250 ng/mL) effect on the endometrial control of trophoblast invasion (Fig. 8). These results were also observed when *ADIPOR1* and *ADIPOR2* were concomitantly invalidated (data not shown).

Discussion

Effective embryo–maternal communication is the key for a successful pregnancy. Adiponectin is a cytokine described to induce the formation of a functional placenta (Benaitreau *et al.* 2010a,b). In this study, we sought to examine the role of adiponectin in maternal compartment by studying its effects on decidualization process and endometrial control of trophoblast invasion.

Figure 6

ADIPOR knockdown in human ESCs. Human ESCs were cultured in DMEM/F12 medium supplemented with E2 and P4 for 13 days. Next, ESCs were cultured for 2 days in the presence of siADIPOR1 (25 nM) or siADIPOR2 (25 nM) or siNS (10 nM). (A and B) Total RNA was extracted after treatment and then quantified by RT-qPCR, as described in the Materials and methods. (A) ADIPOR1 mRNA levels. (B) ADIPOR2 mRNA levels. The data are quoted as the mean $\pm$ s.e.m. of six separate experiments. (C and D) Western Blot analysis of cell lysates (20 μ g) using anti-ADIPOR1 and anti-ADIPOR2 antibodies, as described in the Materials and methods section. (C) ADIPOR1 protein levels were measured in transfected cells. (D) ADIPOR2 protein levels were measured in transfected cells. The figure represents one representative of four experiments. ** $P < 0.01$. Wilcoxon test.

Figure 7

ADIPOR invalidation suppress anti-differentiative adiponectin effect in human ESCs. Human ESCs were cultured in DMEM/F12 medium supplemented with E2 and P4 for 13 days. Next, ESCs were cultured for 2 days in the presence of siADIPOR1 (25 nM) or siADIPOR2 (25 nM) or siNS (10 nM). ESCs transfected with siADIPOR1, siADIPOR2 or siNS were exposed or not to adiponectin (250 ng/mL). Total RNA was extracted after treatment and then quantified by RT-qPCR, as described in the Materials and methods section. The data are quoted as the mean $\pm$ S.E.M. of six separate experiments. (A) Connexin-43 mRNA levels were measured in transfected cells. (B) IGFBP-1 mRNA levels were measured in transfected cells. * $P < 0.05$; NS: non-significant. Wilcoxon test.

Firstly, we attempted to determine whether human ESCs transcribe the *ADIPOR1* and *ADIPOR2* genes. Our results reveal that isolated human ESCs do indeed express these two receptors and strongly suggest that the endometrium is a target tissue for this adipokine. We also observed that *ADIPOR1* and *ADIPOR2* mRNA levels increased moderately throughout the *in vitro* endometrial decidualization (with a 2-fold increase between D0 and D15 of decidualization). Our results differ quantitatively from those observed by Gamundi-Segura and coworkers who observed a 10-fold increase in *ADIPOR* mRNA expression in ESCs during decidualization (Gamundi-Segura *et al.* 2015). As the endometrium undergoes major remodeling over the ovarian cycle, this discrepancy might be due to the time point at which the biopsy was performed

Figure 8

ADIPOR invalidation suppress anti-invasive adiponectin effect in human ESCs. HTR-8/SVneo cells were suspended in the presence of conditioned medium (CM) from ESCs transfected with siADIPOR1, siADIPOR2 or siNS, supplemented or not with adiponectin (250 ng/mL) to perform Transwell invasion assays. The data are quoted as the mean $\pm$ S.E.M. of six separate experiments. * $P < 0.05$; NS: non-significant. Wilcoxon test.

(window of implantation in our study and oocyte pickup in the study by Gamundi-Segura and coworkers). Furthermore, and in accordance with a previous study in human ESCs, we did not observe any regulation of *ADIPOR1* or *ADIPOR2* by adiponectin in these cells (Bohlouli *et al.* 2013). We also found that adiponectin did not seem to be produced by isolated human ESCs under our experimental conditions. This result contrasts with previous publications, including ours (Takemura *et al.* 2006, Dos Santos *et al.* 2012). However, these earlier studies were performed with endometrial tissue that contains various cell types (stromal cells, epithelial cells, blood vessels, etc.). Detection of adiponectin in endometrial tissue might well have been due to the presence of peripheral blood. Experiments to establish whether isolated endometrial epithelial cells express adiponectin are currently underway in our laboratory.

Secondly, we assessed ESC decidualization by measuring the expression of decidualization markers, as prolactin, IGFBP-1 and connexin-43. We observed a time-dependent increased expression of these three markers (between D3 and D15), in the presence of the differentiation medium, indicating that ESCs are fully differentiated at D15. This result is commonly observed for *in vitro* ESC decidualization in the presence of E2 and P4 (Kasahara *et al.* 2001, Godbole *et al.* 2011). We also studied adiponectin effect on ESC decidualization. To this end, we used subphysiological doses of recombinant human adiponectin (25 ng/mL and 250 ng/mL), which are much lower than those observed in the human circulation. This recombinant human adiponectin effectively mainly

comprises the high-molecular-weight form (Bub *et al.* 2006), considered to be the active isoform (Kobayashi *et al.* 2004). Moreover, adiponectin is commonly used at these concentrations for *in vitro* experiments with various tissue and cell types (Bråkenhielm *et al.* 2004, Dos Santos *et al.* 2008, Duval *et al.* 2016). Our results clearly show that adiponectin negatively controls biochemical ESC decidualization in a dose-independent manner. We effectively observed a significant decrease of prolactin secretion in ESCs treated with adiponectin. There was no effect on prolactin mRNA expression. This lack of correspondence between RNA and protein levels is not surprising and has been already described (Salari *et al.* 2012, Beileke *et al.* 2015). One study specifically evidenced the mismatch between the prolactin mRNA expression and hormone release (Castaño *et al.* 1997).

Thirdly, we demonstrated that adiponectin downregulates *IGFBP-1* and *connexin-43* mRNA expression levels. These effects were mainly observed when ESCs were fully differentiated (after 15 days of decidualization). As levels of *ADIPOR* mRNA doubled between D0 and D15 of decidualization, this observation might explain the delayed, negative effect of adiponectin on decidualization. Taken as a whole, these data suggest that like hCG, leptin and interleukin-1, adiponectin could be a new regulator of human ESC decidualization (Kariya *et al.* 1991, Kasahara *et al.* 2001, Tanaka *et al.* 2003).

As one of the major roles of decidual cells is the control of trophoblast invasion (Godbole *et al.* 2011, Sharma *et al.* 2016), we next looked at whether adiponectin could modify the ability of decidualized ESCs to regulate this process. Therefore, we used Transwell assays to estimate the trophoblast HTR-8/SVneo cell line invasive capacities when seeded with CM from ESCs treated or not with adiponectin. Our results clearly demonstrate that CM from ESC cultured in the presence of adiponectin significantly decreased trophoblastic cell ability to invade Matrigel. In order to gain more information at molecular level, we next investigated adiponectin effect on the MMP/TIMP balance in human ESCs. Our data demonstrate that adiponectin significantly and specifically upregulated *TIMP-3* mRNA expression and, conversely, reduced MMP-2,-9 activities in ESCs. Thus, it seems that in human endometrium, adiponectin can modulate, at least in part, ECM remodeling *via* regulation of the MMP-2,-9/TIMP-3 balance. Adiponectin effects on human ESCs were once again dose independent, but is not surprising, since the majority of *in vitro* studies performed in various cell types such as placental cells, did not show a dose-response effect

for adiponectin (Benaitreau *et al.* 2009, Duval *et al.* 2016). Taken as a whole, these data suggest that adiponectin induces the production of endometrial factors, which could limit trophoblast invasion. At present, it is difficult to precise whether the secretion of endometrial factors is directly controlled by adiponectin, or indirectly *via* the induced production of hormones or cytokines. A recent study effectively demonstrated that TNF- α facilitates EVT invasion *in vitro* by regulating the MMP/TIMP balance in human ESCs (Haider & Knöfler 2009). Thus, endometrial TNF- α signal could be modified by adiponectin, as described in macrophages and in adipocytes (Tsatsanis *et al.* 2005, Greenberg & Obin 2006). Additional experiments will be needed to clarify this hypothesis.

In order to confirm the specific involvement of ADIPOR1 and ADIPOR2 in adiponectin regulation of ESC decidualization and the control of trophoblast invasion, we silenced ADIPORs in ESCs. We found that *ADIPOR1* and *ADIPOR2* knockdown (separately or concomitantly) suppressed adiponectin anti-differentiative and anti-invasive effects in human ESCs. These results clearly demonstrated the critical involvement of the ADIPORs in adiponectin control of ESC functions.

Lastly, we also attempted to determine whether or not the human ESC decidualization status is associated with the cell ability to control ECM remodeling. We clearly demonstrated that *TIMP* expression and MMP activities are closely related to ESC decidualization status. Indeed, we confirmed that *TIMP-3* mRNA expression significantly rose throughout decidualization, as described by Zhang and Salamonsen (Zhang & Salamonsen 1997). We also found that non-decidualized ESCs secrete an active MMP, which might be MMP-3. Indeed, MMP-3 is a 45 kDa protein able to digest gelatin. However, the putative MMP-3 activity falls during decidualization. With regard to MMP-9, and more especially MMP-2, which appears to be the most abundantly produced gelatinase in ESCs, we observed an upregulation of their activities during decidualization. These results confirmed that ECM remodeling occurs during ESC differentiation in order to maintain endometrial integrity, which is essential for embryo implantation.

Interestingly, we noted that although adiponectin enhances the MMP/TIMP ratio in human trophoblastic cells, and thus, promotes invasion (Benaitreau *et al.* 2010a); this adipokine decreases the MMP/TIMP ratio in human ESCs during cell decidualization. This would limit cell migration and consequently avoid excessive invasion. Taken as a whole, these data strongly suggest

that the adiponectin system has a critical role in the establishment of embryo implantation by maintaining the balance of pro-invasive and anti-invasive signals in placenta and endometrium, respectively. In conclusion, it should be noted that dysregulation of the placental invasive potential is associated with several pregnancy pathologies, as spontaneous miscarriages, preeclampsia, intrauterine growth restriction (reduced trophoblast invasion), placenta accreta and choriocarcinoma (excessive trophoblast invasion) (Lala *et al.* 2002, Anin *et al.* 2004, Ball *et al.* 2006, Kadyrov *et al.* 2006). Hence, our results suggest that adiponectin, through dual control in trophoblast and decidual cells, could have a causative role in the appearance of these pathologies by modulating the invasive capacities.

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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Author contribution statement

F D and M N D designed the experiments. F D and H M performed the experiments. F D and M N D analyzed the data. F D, M N D and E D S wrote the manuscript. N S B provided human endometrial biopsies for experiments. V S performed automated quantifications. F V participated in critical revision of the manuscript.

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