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RESEARCH ARTICLE

The effect of obesity on uterine receptivity is mediated by endometrial extracellular vesicles that control human endometrial stromal cell decidualization and trophoblast invasion

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Abstract

The objectives of the present study were to determine whether obesity impacts human decidualization and the endometrial control of trophoblast invasion (both of which are required for embryo implantation) and evaluate the potential involvement of endometrial extracellular vesicles (EVs) in the regulation of these physiological processes. Using primary human cell cultures, we first demonstrated that obesity is associated with significantly lower in vitro decidualization of endometrial stromal cells (ESCs). We then showed that a trophoblastic cell line's invasive ability was greater in the presence of conditioned media from cultures of ESCs from obese women. The results of functional assays indicated that supplementation of the culture medium with EVs from nonobese women can rescue (at least in part) the defect in in vitro decidualization described in ESCs from obese women. Furthermore, exposure to endometrial EVs from obese women (vs. nonobese women) was associated with significantly greater invasive activity by HTR-8/SVneo cells. Using mass-spectrometry-based quantitative proteomics, we found that EVs isolated from uterine supernatants of biopsies from obese women (vs. nonobese women) presented a molecular signature focused on cell remodelling and angiogenesis. The proteomics analysis revealed two differentially expressed proteins (fibronectin and angiotensin-converting enzyme) that might be involved specifically in the rescue of the decidualization capacity in ESCs from obese women; both of these proteins are abundantly present in endometrial EVs from nonobese women, and both are involved in the decidualization process. In conclusion, our results provided new insights into the endometrial EVs' pivotal role in the poor uterine receptivity observed in obese women.

KEYWORDS

decidualization, embryo implantation, extracellular vesicle, human endometrium, obesity, proteomics, trophoblast invasion

1 | INTRODUCTION

The prevalence of obesity (defined as a body mass index [BMI] of 30 kg/m^2 or over) has increased substantially over the last several decades. This metabolic disorder is a worldwide public health concern because it predisposes the person to a large number of diseases and disorders, including infertility. Although many obese women conceive spontaneously, obesity is associated with abnormally low clinical pregnancy rates. Specifically, obese women are three times more likely to suffer from anovulatory infertility than women with a normal BMI (Rich-Edwards et al., 1994). Even when an obese woman does ovulate, the time to conception is lengthened (e.g., by a factor of two in overweight women) (Hassan & Killick, 2004). Furthermore, obesity has a negative impact on the outcomes of assisted reproduction programs, with higher miscarriage rates, lower live birth rates, and lower embryo implantation rates when compared with normal-weight women (Fedorcsák et al., 2004; Lintsen et al., 2005; Maheshwari et al., 2007; Provost et al., 2016; Wang et al., 2000).

Embryo implantation is a key step in the successful establishment of pregnancy. The required dialog between the competent embryo and the receptive endometrium is complex (Paria et al., 2002). In humans, endometrial receptivity is a cyclic process initiated independently of pregnancy and which is spatially and temporally restricted to the secretory phase of the menstrual cycle known as the "implantation window" (days 20–24 of the ideal 28-day cycle). Uterine receptivity is associated with many morphological and biochemical changes in endometrial cells in response to the ovarian steroids 17 β-estradiol (E2) and progesterone (P4). More specifically, these changes include the transformation of stromal fibroblasts into specialized round, relatively large, epithelioid-like or polygonal secretory decidual cells (Gellersen et al., 2007). Prolactin (PRL) and insulin-like growth factor binding-1 (IGFBP-1) are secreted by fully differentiated endometrial stromal cells (ESCs) and are commonly monitored as biochemical markers of human stromal cell differentiation (Dunn et al., 2003). During decidualization, ESCs progressively acquire migratory and invasive properties, in order to facilitate trophoblast infiltration into the endometrial stroma and then placentation (Grewal et al., 2008). Trophoblast invasion control by decidual cells is realized by the ESCs' secretion of two matrix metalloproteinases (MMPs-2 and -9), which degrade the major component of the endometrial extracellular matrix (*i.e.* collagen IV). The level of MMP activity is regulated by specific tissue inhibitors of metalloproteinases (TIMPs), which bind to and inactivate MMPs with a 1:1 stoichiometry (Huppertz et al., 1998). Correct regulation of the MMP/TIMP balance in ESCs is thus essential for successful embryo implantation (Nissi et al., 2013). Defective decidualization impedes the endometrium's ability to receive and sustain the embryo and therefore is associated with reproductive disorders such as preeclampsia, recurrent miscarriages, and intra-uterine growth restriction (Dunk et al., 2019).

Research by our laboratory and others has revealed that decidualization process is regulated by signalling molecules like prostaglandin E2, transforming growth factor β1, dehydroepiandrosterone, adiponectin, and preimplantation factor (Duval et al., 2017; Gibson et al., 2018; Kane et al., 2010; Okada et al., 2018; Santos et al., 2021). It has recently become clear that extracellular vesicles (EVs, including multivesicular-body-derived vesicles [exosomes] and plasma-membrane-derived vesicles [ectosomes, microvesicles, and microparticles]) have a key role at the fetal-maternal interface and in the decidualization process (Homer et al., 2017; Rodriguez-Caro et al., 2019). These EVs are released from the cell surface *via* exocytosis or budding by the vast majority of cell types, including those in the endometrium (Ng et al., 2013). EVs contains many cell-type-specific, functionally active molecules but have broadly been found to include proteins, cytokines, lipids, nucleic acids (RNA and DNA), and metabolites that originate from the parent cells (Greening et al., 2016). Thus, the contents of EVs reflect the cell origin and can also be affected by underlying diseases and disorders. Human endometrial fluid contains miRNAs whose target genes are involved in successful embryo-crosstalk during implantation; these genes variously code for adherence junctions, extracellular matrix receptors, and components of the vascular epidermal growth factor-signalling pathway, the Janus kinase/signal transducer and activator of transcription signalling pathway, and the Toll-like receptor signalling pathway (Ng et al., 2013). Furthermore, prostaglandins E_2 and $F\alpha_{2,}$ (which are particularly abundant during the implantation window) are relatively abundant in uterine EVs and might help to sustain the blastocyst at the time of embryo implantation (Vilella et al., 2013).

Several studies have addressed the EVs' role in the modulation of various pathophysiological processes in obesity, diabetes mellitus, cancer, and placental dysfunction (Pardo et al., 2018). For example, it has been shown that plasma EV levels are 10-fold higher in obese people than in normal-weight people (Eguchi et al., 2016). Furthermore, obesity appears to alter the cargo of EVs secreted by adipocytes; these vectors were enriched in molecules involved in inflammation, immune efficiency, and systemic insulin resistance (Lakhter & Sims, 2015). To the best of our knowledge, the number and content of EVs secreted by the endometrium of obese women have not been determined.

Although the literature data on whether obesity has a significant effect on the endometrium are contradictory, more recent studies have found that obesity impairs uterine functions (Broughton & Moley, 2017). Bellver et al.'s clinical experience (9587 first cycles of oocyte donation with normal-weight donors and recipients classified according to their BMI) suggested that female obesity worsens the reproductive outcome of assisted reproduction programs - probably due to lower uterine receptivity (Bellver et al., 2013). This observation was confirmed by the results of Provost *et al.*'s large cohort study (Provost et al., 2016). Furthermore, two transcriptomic analyses of human endometrial samples collected during the implantation window demonstrated that relative to nonobese individuals, obesity alters the expression of genes primarily involved in inflammation, immune system activity, and protein-binding molecular functions (Bellver et al., 2011; Comstock et al., 2017). Recent studies of animal models have confirmed

that increased adiposity impairs uterine receptivity *via* morphological and functional changes in the uterus; ectopic lipid accumulation and macrophage infiltration are associated with lipotoxicity, local inflammation, and oxidative stress activation (Bazzano et al., 2018, December; Gao et al., 2021; Sessions-Bresnahan et al., 2018, October). Lastly, in vitro experiments performed with immortalized and primary human ESCs showed clearly that exposure to palmitic acid (the most abundant unsaturated fatty acid in the modern Western diet) suppressed decidualization in a statistically significant, dose-dependent manner (Rhee et al., 2016). Overall, the molecular mechanisms underlying obesity's impact on uterine receptivity in general and decidualization in particular are complex and have not yet been clearly elucidated.

The objectives of the present study were to determine whether decidualization and the endometrial control of trophoblast invasion are compromised in the endometrium of obese women and whether EVs have a role in these impairments. To this end, we compared between obese women and nonobese women in terms of (i) the in vitro decidualization of ESCs, (ii) the control of in vitro trophoblastic invasive abilities by ESCs, and (iii) quantitative and qualitative aspects of endometrial EV contents. We also used functional assays to probe the effects of medium supplementation with endometrial EVs on decidualization in vitro and to assess the endometrial EVs' involvement in the control of trophoblast invasion in women.

2 | MATERIALS AND METHODS

2.1 | The study population and tissue collection

A total of 23 infertile, normally cycling women (age: 27–40) undergoing a biopsy for fertility evaluation were included in the present study. Endometrial tissue was obtained during the putative implantation window. All study participants had undergone a diagnostic work-up. We excluded women with endocrine dysfunctions, polycystic ovarian syndrome (PCOS), autoimmune disease, infectious diseases, uterine malformations (including submucous fibroids or polyps, intramural fibroids > 4 cm, clinical endometriosis, hydrosalpinx, or a low (<2 cm) endometrial volume), haemostasis disorders, chromosomal alterations, a history of recurrent pregnancy loss (≥3 losses before the 14th week of gestation), and tobacco consumption (>10 cigarettes/day, which is known to significantly reduce uterine receptivity) (Soares et al., 2007). Patients were classified according to the World Health Organization's criteria for normal-weight (a BMI between 18.5 and 25 kg/m^2) and obesity (a BMI ≥30 kg/m^2) (Report of a WHO consultation, 2000).

The study was approved by the local investigational review board (*CPP Ile-de-France*, Paris, France; approval reference: 13028). All participants provided their written informed consent before tissue sampling.

2.2 | Human ESC cultures

All the human endometrial biopsies were collected by the same practitioner, in order to ensure size reproducibility (2 mm in diameter, 1.5 cm in length). The biopsies were maintained in DMEM/F12 medium at 4°C until the isolation of ESCs. Human ESC isolation and culture were performed as described by Gonzalez et al. (1999). Briefly, tissue samples were minced into small pieces and digested in a two-step process. Firstly, tissues were incubated for 1 h at 37°C in phenol-red-free DMEM/F12 medium supplemented with DNase type I (0.0005%), penicillin (10 μg/mL), streptomycin (100 U/mL) (Gibco/ThermoFisher Scientific, Waltham, MA) and collagenase (0.1%) (Boehringer, Ingelheim, Germany). The supernatant was filtered through a 100 μm nylon screen and then centrifuged at 200 g for 10 min. A second enzymatic digestion was performed on undigested tissue for 10 min at 37°C in DMEM/F12 medium supplemented with trypsin (0.25%) (Difco Laboratories, Detroit, MI), 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 200 g for 10 min. Cell pellets from both digestions were pooled and centrifuged at 200 g for 10 min.

Cells were seeded at a density of 1×10^5 per cm^2 and cultured in DMEM/F12 medium supplemented with streptomycin (10 μg/mL), penicillin (100 U/mL) and fetal calf serum (FCS, 10%) (Gibco/ThermoFisher Scientific) at 37°C in a 5% CO_2/95% air atmosphere. When the cells reached confluence, they were cultured in differentiation medium (DMEM/F12 supplemented with E2 (10^{-8} M), P4 (10^{-6} M) (Gibco/ThermoFisher Scientific), charcoal-stripped FCS (2%), penicillin (10 μg/mL) and streptomycin (100 U/mL)) for 14 days. The medium was changed every 2 days, as described previously (Duval et al., 2017).

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

The influence of ESC-secreted molecules on trophoblastic cell invasiveness was assessed using the HTR-8/SVneo immortalized extravillous trophoblast (EVT) cell line (kindly provided by Dr Nadia Alfaidy (CEA, Grenoble, France) and in agreement with Dr Charles Graham (Queen's University, Kingston, ON, Canada)). HTR-8/SVneo cells were cultured in Roswell Park Memorial Institute (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 (Transwell®, Greiner Bio-One SAS, Courtaboeuf, France), according to the protocol described by Tapia-Pizarro et al. (2013). The HTR-8/SVneo cells (3×10^4 per cm^2) were then suspended in 250 μL of conditioned medium (CM) from 14-day-decidualized ESCs obtained from obese women or nonobese women. In order to validate our experimental conditions, RPMI medium supplemented with E2 (10^{-8} M), P4 (10^{-6} M) and (in some experiments) a positive control (adiponectin, 250 ng/mL) was added at the bottom well. RPMI medium supplemented with FCS (10%) medium 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 on the lower surface of the insert were washed off 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 (a Leica white light laser TCS SP8-X, Leica Microsystems, Wetzlar, Germany). On each insert, invasive cells were counted on five randomly selected fields, using the post-imaging procedure in ImageJ software (NIH, Bethesda, MD). Invasive cells were defined as those whose nucleus was larger than 8 μm (i.e., the pore size).

2.4 | Zymography

After 14 days of culture with differentiation medium containing steroid hormones, total gelatinase activities from ESCs isolated from obese or nonobese women were analyzed using zymography. Aliquots of CM containing 60 μg of protein were resolved under non-reducing conditions in 10% polyacrylamide gels containing 1 mg/mL gelatin (Gibco/ThermoFisher Scientific). The gels were washed in Triton X-100 (2.5%) for 30 min (to remove SDS), incubated overnight at 37°C in a renaturing buffer (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl, and 0.02% sodium azide), 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 the proteolytic activity was quantified using ImageJ software.

2.5 | Isolation of EVs

Human endometrial biopsies have been transported and maintained in DMEM/F12 medium at 4°C. Serum-free medium was used, in order to avoid contamination by FCS. After 4 h of contact with the endometrial biopsies, EVs were isolated from the supernatants of endometrial biopsies. Supernatants were centrifuged at 200 ***g*** for 10 min (to remove any cells in suspension), and stored at -80°C until required for our experiments. The EVs were isolated from the supernatants via single-step size exclusion chromatography (qEV original 70 nm column, Izon Science Ltd, Christchurch, New Zealand). The column was washed with PBS Dulbecco's PBS (Sigma-Aldrich Merck, Darmstadt, Germany). The supernatant was loaded onto the column and then eluted with 6 mL of PBS (12 fractions of 0.5 mL). Transmission electronic microscopy showed that fractions 7–10 contained EVs. Hence, for each biopsy, fractions 7–10 were pooled and then ultracentrifuged at 100,000 ***g*** for 1 h at 4°C (LE-80K ultracentrifuge, Beckman, Brea, CA) with a SW60 rotor. The supernatants were removed, and the pellet resuspended in 50 μL of PBS.

2.6 | Observation of EVs, using transmission electronic microscopy

Materials were directly adsorbed onto a carbon film membrane on a 300-mesh copper grid, stained with 1% uranyl acetate, dissolved in distilled water, and dried at room temperature.

Grids were examined with a Hitachi HT7700 electron microscope operating at 80 kV (Elexience, Verrières-le-Buisson, France). Images were acquired with a charge-coupled device camera (AMT).

2.7 | Extracellular vesicle size and concentration analysis

The EVs' size distribution and concentration were measured with nanoparticle tracking analysis (NTA) on a ZetaView system equipped with a 488 nm laser (Particle Metrix GbmH, Inning am Ammersee, Germany). In NTA, a liquid suspension's Brownian motion and light scattering properties provide information on the particle size distribution. For each experiment, dilutions at eleven different positions were analyzed. The particle detection was carried out in the sample cell with following specifications and analysis parameters: sensitivity, 80; shutter, 100; max area, 1000; min area, 10; min brightness, 20. NTA software (version 8.05.05, SP2) was used to capture and analyze the data. The samples were diluted as a function of the input sample concentrations. The NTA system's optimal working range for the EV concentrations was 1×10^6 to 1×10^9 particles per ml. Videos (30 frames/sec) were recorded, and the results were validated when around 500 valid tracks had been obtained for each run.

2.8 | Proteomic analyses

2.8.1 | Sample preparation

Vesicles were disrupted in Laemmli buffer overnight at room temperature. Proteins were loaded on SDS-PAGE gels (short migration). Gel bands were cut out; proteins were reduced with dithiothreitol (10 mM) for 30 minutes at 56°C and then alkylated with iodoacetamide (final concentration: 55 mM) for 45 min at room temperature in the dark. In-gel digestion was based on 50 mM ammonium bicarbonate (pH 8.0) overnight at 37°C, with 200 ng trypsin (Promega, Madison, WI) per sample. Peptides were extracted with 5% formic acid in 50% acetonitrile in water (v/v). The supernatant and the extracted tryptic peptides were dried and resuspended in 30 μL of 0.1% (v/v) formic acid/2% (v/v) acetonitrile.

2.8.2 | Mass spectrometry

Proteins in the samples obtained from four obese women and seven non-obese women were identified using an Orbitrap Fusion Lumos Tribrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled to an UltiMate3000 RSLCnano ultraHPLC system (Dionex, Sunnyvale, CA). Each 4 μL sample was injected for online desalting onto a PepMap C-18 reverse-phase (RP) nanotrap column (3 μm, 75 μm × 20 mm, Dionex) with nanoViper fittings (flow rate: 20 μL min^{-1}), separated on a PepMap C-18 reverse-phase nano column (3 μm, 75 μm × 50 cm), and eluted with a 50 min gradient of 5%–30% acetonitrile in 0.1% formic acid at 300 nL min^{-1}, a 5-min ramp to 40% acetonitrile/0.1% formic acid, a 3-min ramp to 98% acetonitrile/0.1% formic acid, and a 5-min hold at 98% acetonitrile/0.1% formic acid. The mass spectrometer was operated in positive ion mode, with the nano spray voltage set to 1.6 kV and the source temperature set to 270 °C.

An internal calibration (lock mass) was performed using the background polysiloxane ion signal, with mass-to-charge ratios (m/z) of 445.12002, 519.13882, 593.15761, and 667.17640. The instrument was operated in data-dependent acquisition mode and high-energy collisional dissociation fragmentation mode (collision energy: 30%). In all experiments, full MS scans were acquired over the 400–1500 m/z mass range, with detection in the Orbitrap mass analyzer at a resolution of 120,000, an automatic gain control set to 1×10^5 and an intensity threshold of 20,000. Each precursor ion scan was followed by a 3-s "top speed" data-dependent Orbitrap MS/MS run, with a 1.6 m/z window for the quadrupole isolation of precursor peptides with multiply charged ions from 2 to 4.Fragment ion spectra were acquired in the Orbitrap mass analyzer at a resolution setting of 30,000, an automatic gain control set to 5×10^4, and a maximum injection time of 54 ms. Polysilaxolane ions m/z 445.12002, 519.13882 and 593.15761 were used for internal calibration.

2.8.3 | Bioinformatics: protein identification and quantification

The data were converted into mzXML format using MS convert (ProteoWizard, version 3.0.8934). Proteins were identified using X!Tandem v.2017.2.1.4 (Craig & Beavis, 2004) by matching peptides against the Swiss-Prot *Homo sapiens* database (https://www.uniprot.org/, version 2021, containing 20,396 entries). Proteins were filtered and grouped using open-source X!TandemPipeline software (version 0.4.37, http://pappso.inrae.fr/bioinfo/xtandempipeline/) (Langella et al., 2017). To eliminate spectra due to contaminants, the data were compared with a contaminant database.

The proteome identification process was run with a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 10 ppm. The trypsin digestion rule ("after Arg and Lys, except before Pro") was applied, and semi-enzymatic cleavage was ruled out. The fix modification was set to cysteine carbamidomethylation, and methionine oxidation was considered to be a potential modification. In a second pass, N-terminal acetylation was added as another potential modification, whereas all the other above-mentioned settings remained unchanged. Identified proteins were filtered as follows: (i) peptide E-value <0.01 and a minimum of two peptides per protein, and (ii) a protein E-value of 10^{-4}. The false discovery rate was estimated to be 0.14% for peptides and 0.1% for proteins.

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium database (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository, with the dataset identifier PXD039568.

2.8.4 | The spectral count (SC) and the extracted ion chromatogram (XIC)

Two protein quantification methods were used to detect changes in protein abundance. As recommended by Blein-Nicolas and Zivy, we first used the SC to detect semi-quantitative changes and presence/absence variations (Blein-Nicolas & Zivy, 2016). Protein abundances were calculated from the SC of independent biological replicates and from the pool of samples

used for functional assays (see the sample design in Supplementary Figure 1) (Carvalho et al., 2008; Usaite et al., 2008). We then used a more precise XIC-based approach to detect smaller abundance changes. MassChroq software (version 2.2.12, http://pappso.inrae.fr/bioinfo/masschroq/) was used for retention time alignment and precursor peak intensity integration of the XIC. The MassChroqR tool box (https://forgemia.inra.fr/pappso/mcqr version 0.3.7, http://pappso.inrae.fr/) was used to check the proteomic data quality (peak width, retention time variation, principal component analysis, etc.), eliminate dubious samples, and apply statistical tests.

In the SC analysis, we removed proteins with less than five spectra in all the injections. The ratio between the minimum and maximum mean abundance values was computed for each factor or combination of factors of interest.

The XIC data were normalized in order to remove systemic bias between samples and more complex variations resulting from transient stochastic events during LC-MS/MS run, such as ESI instability. Missing peptide ion XIC data were imputed according to the correlation between peptides from the same protein. The relative abundance of proteins was determined by summing the values for at least two specific peptides.

After the abundances had been $\log_{10}$-transformed, the statistical significance of between-group differences was determined in an analysis of variance. The resulting p-values were Benjamini-Hochberg corrected for multiple testing (Benjamini & Hochberg, 1995). After adjustment, the threshold for statistical significance was set to $p < 0.05$ for the SC data and 0.01 for the XIC data. Lastly, proteins with a fold-difference (obese vs. normal-weight women) above 1.5 or below 0.66 were considered to have been significantly affected.

2.9 | Immunoblots

To check for the presence of EVs in our preparations, we performed Western blotting with the EV-specific markers CD9, CD81, and HSP70. All the samples were suspended in non-reducing buffer (LDS Sample Buffer [4×], Invitrogen/ThermoFisher Scientific, Waltham, MA) and warmed for 10 min at 70°C. Protein samples were separated using SDS-PAGE (NuPAGE Bis-Tris gradient 4%–12% gels, Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Next, the membrane was blocked at room temperature with 5% bovine serum albumin (BSA) + 0.05% Tween for 1 h. The membranes were then exposed to rabbit primary antibodies against CD9, CD81 or hsp70 (1:1000 in 5% BSA + 0.05% Tween tris-buffered saline (TBS), EXOAB-KIT, System Biosciences, Palo Alto, CA) at 4°C. After overnight incubation, the membranes were washed extensively with 0.05% Tween TBS and incubated with a horse radish peroxidase-conjugated goat anti-rabbit secondary antibody (EXOAB-KIT, SBI; 1:20000 in 5% BSA + 0.05% Tween TBS). After a final wash, chemiluminescence substrate (ECL Plus, Pierce-ThermoFisher Scientific, Waltham, MA) was added to the membranes. The membranes were imaged with the ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA).

2.10 | Functional assays with EVs

After 7 days of exposure to differentiation medium (DMEM/F12 supplemented with E2 (10^{-8} M), P4 (10^{-6} M), charcoal-stripped FCS (2%), penicillin (10 μg/mL) and streptomycin (100 U/mL)), cells isolated from obese women were treated for 48 h with PBS (as a control) or EVs (5×10^{10} EVs/10^6 cells) isolated from the endometrial secretions of five nonobese women (see the sample design in Supplementary Figure 1). The differentiation medium was changed every 2 days during the 14-day cell differentiation period. Lastly, cell supernatants were collected for the PRL secretion assay, and total RNA was extracted for RT-qPCR analyses.

The trophoblastic cell invasiveness in response to EV supplementation of the culture medium was measured in a similar way to the above-described Matrigel® assay. Invasion assays were performed in 24-well plates containing Matrigel®-coated polycarbonate membrane (pore size: 8 μm) invasion chamber inserts. HTR-8/SVneo cells were suspended (3×10^4 per cm^2) in 250 μL of RPMI medium supplemented with penicillin (10 μg/mL), streptomycin (100 U/mL) and (in some experiments) EVs isolated from endometrial secretions of five nonobese women or five obese women (5×10^{10} EVs/10^6 cells). In order to validate our experimental conditions, 250 ng/mL adiponectin was used as a positive control (Duval et al., 2017).

2.11 | Reverse-transcription - quantitative polymerase chain reactions (RT-qPCRs)

ESCs (1×10^5 per cm^2) were seeded in 12-well culture plates. After 14 days of differentiation, total RNA (0.1 μg) from the ESCs was extracted and reverse-transcribed, as described previously (Benaitreau et al., 2009). Quantitative PCR was performed using the C1000 Thermal Cycler (CFX96 real-time system; Bio-Rad Laboratories Inc.) and the primer sets indicated in Table 1. The final primer concentration was 25 μM. The three reference genes (coding for ribosomal protein L13A (RPL13A), TATA box-binding protein (TBP) and β2-microglobulin) were chosen for the reasons described previously (Santos et al., 2021). For each sample, the concentration ratios (target mRNA/three reference mRNAs) were calculated using CFX Manager software (version 3.0, Bio-Rad) and expressed in arbitrary units, as described previously (Santos et al., 2021).

TABLE 1 Primers used in RT-PCR assays.

Primer set	Sequence	PCR product (bp)
PROLACTINSenseAntisense	**5'** AGC CAG GTT CAT CCT GAA A **3'** **5'** TTC TCA GAG CGG AAA GAC GA **3'**	99
IGFBP-1 SenseAntisense	**5'** ATC ACA GCA GAC AGT GTG AGA **3'** **5'** CCA CGC AGA TGG GAA CCT TA **3'**	71
TIMP-1 SenseAntisense	**5'** GGG CTT CAC CAA GAC CTA CA **3'** **5'** TGC AGG GGA TGG ATA AAC AG **3'**	71
TIMP-2 SenseAntisense	**5'** GAA GAG CCT GAA CCA CAG GT **3'** **5'** GGG GGA GGA GAT GTA GCA C **3'**	85
TIMP-3 SenseAntisense	**5'** CAC CTC TCC ACG AAG TTG C **3'** **5'** AGC TTC CGA GAG TCT CTG TG **3'**	117
TBPSenseAntisense	**5'** TGC ACA GGA GCC AAG AGT GAA **3'** **5'** CAC ATC ACA GCT CCC CAC CA **3'**	132
***β*2-microglobulin**SenseAntisense	**5'** TGC TGT CTC CAT GTT TGA TGT ATC T **3'** **5'** TCT CTG CTC CCC ACC TCT AAG T **3'**	86
RPL13A SenseAntisense	**5'** CCT GGA GGA GAA GAG GAA AGA GA **3'** **5'** TTG AGG ACC TCT GTG TAT TTG TCA A **3'**	125

2.12 | Prolactin secretion

The secretion of PRL into the culture medium after 7 and 14 days of cell decidualization was measured using an automated immuno chemiluminescence assay system (Kryptor®, B.R.A.H.M.S., Saint-Ouen, France). The level of PRL secretion was expressed per 1 μg of total protein (measured using Bradford's method, with BSA as the standard.

2.13 | Lactate dehydrogenase secretion

The cytotoxicity of EVs was measured by assaying the level of lactate dehydrogenase (LDH) activity released into the culture medium, as described previously (Morice et al., 2011).

2.14 | Statistical analyses

Statistical analyses were performed on the raw data from 3 to 14 separate experiments. A non-parametric, unpaired Mann-Whitney test was used to compare values for the obese *vs*. nonobese groups (GraphPad Prism 9). Details of the statistical tests applied to the proteomic data are given above in the corresponding section.

3 | RESULTS

3.1 | The impact of obesity on human ESC decidualization

We used two well-established biochemical markers of decidualization (PRL and IGFBP-1) to study obesity effects on decidualization. After 14 days of cell decidualization, *PRL* and IGFBP-1 mRNA expression levels were significantly lower in ESCs isolated from obese women than in ESCs isolated from nonobese women (with relative decreases of 77% and 80% for *PRL* and IGFBP-1, respectively; $p < 0.05$) (Figure 1). Similarly, PRL secretion was lower in ESCs isolated from obese women (a relative decrease of 63% vs nonobese women; $p < 0.05$) after 14 days of decidualization but not after 7 days.

3.2 | The impact of obesity on the endometrial control of trophoblast invasion

In order to determine whether excess adiposity perturbs the endometrial control of trophoblast invasion, HTR-8/SVneo immortalized EVT cells were cultured in the presence of CM conditioned by fully differentiated ESCs (Figure 2a). The invasive activity of HTR-8/SVneo cells (a relative increase of 24%; $p < 0.05$) was greater after exposure to CM from ECSs isolated from obese women than for those isolated from nonobese women (Figure 2b,c).

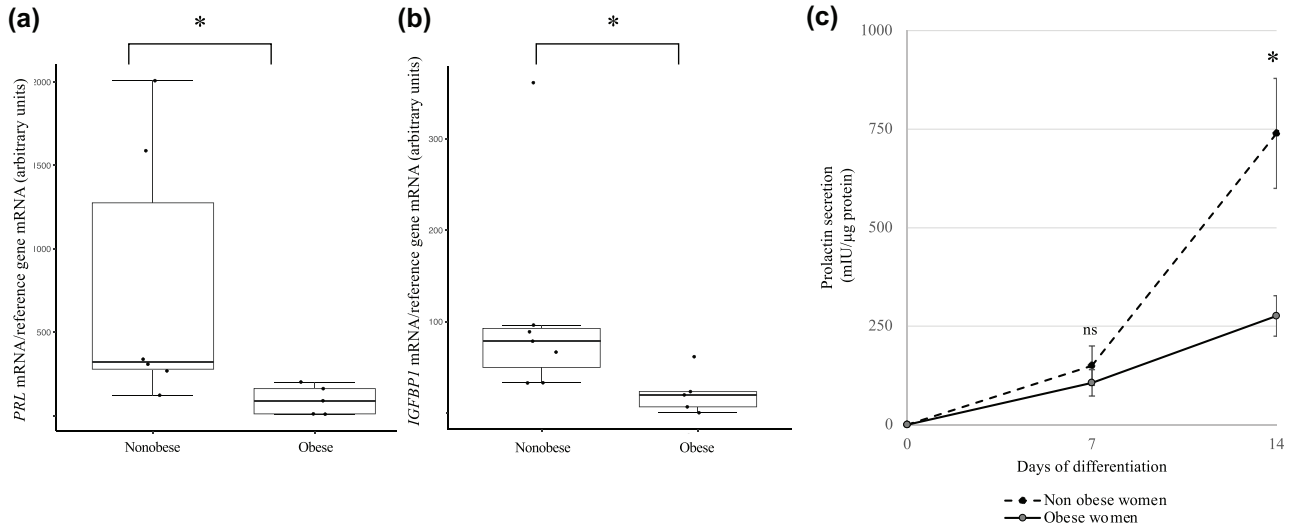

FIGURE 1 The effect of obesity on human ESC in vitro decidualization. Human ESCs were cultured for 14 days (D) in DMEM/F12 medium supplemented with E2 and P4. (a and b) Total RNA was extracted on D14. *PRL* (a) and *IGFBP-1* (b) mRNA expression levels were quantified using RT-qPCR assays. The data are presented as box plots of five to seven separate experiments. (c) Prolactin secretion into the endometrial supernatants was measured on D7 and D14. The data are presented as the mean ± SEM of 7 to 14 separate experiments. $^*p < 0.05$ (Mann-Whitney test) for obese women *vs.* nonobese women, ns: nonsignificant.

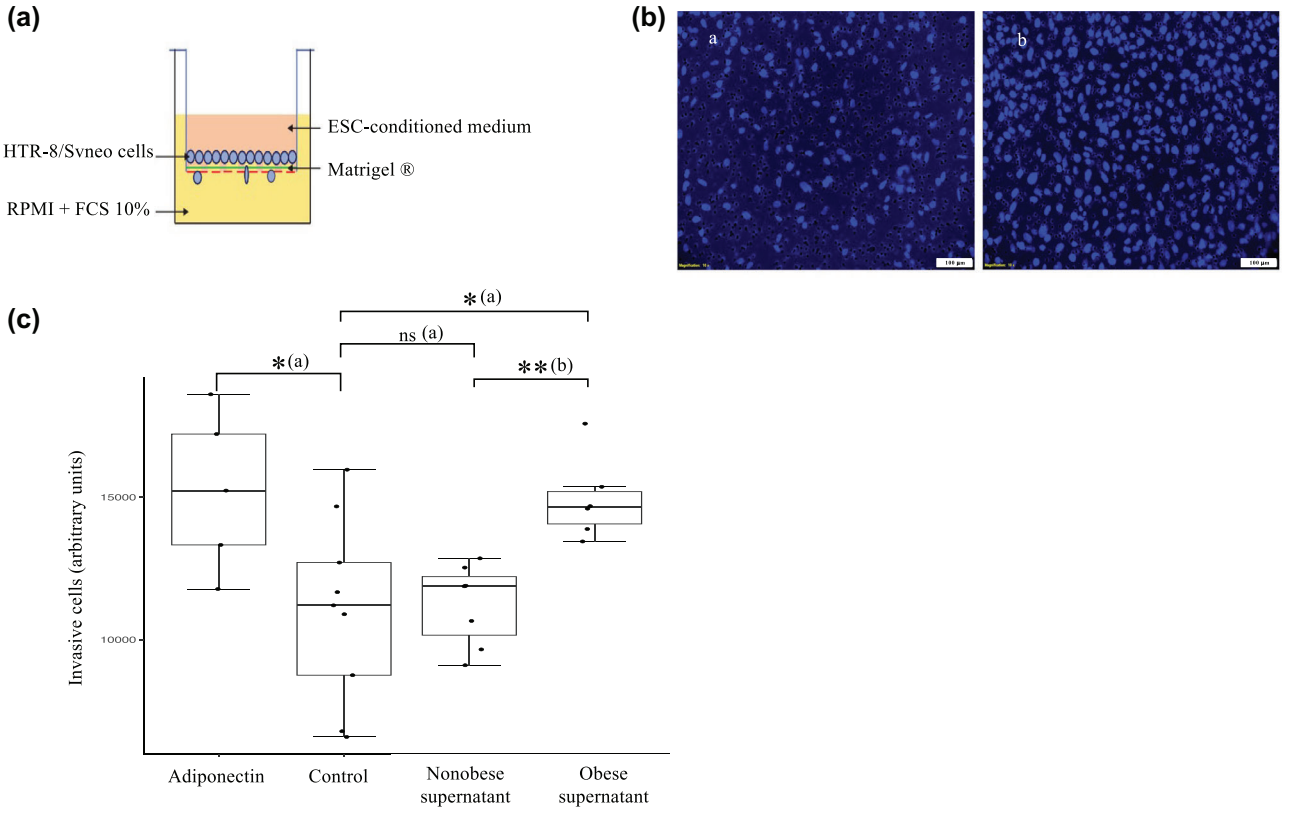

FIGURE 2 The effect of obesity on human endometrial control of trophoblast invasion. (a) Transwell® migration assays were performed with HTR-8/SVneo cells. (b) Representative microphotographs from nine separate experiments, showing fixed and Hoechst-stained HTR-8/SVneo cells on the bottom side of the Transwell® membrane (magnification: ×10). HTR-8/SVneo cells were cultured for 48 h with conditioned medium from decidualized ESCs isolated from nonobese women ((a)) or obese women ((b)). (c) HTR-8/SVneo cells were suspended with conditioned medium from decidualized ESCs isolated from nonobese women or obese women or with control medium supplemented (or not) with the positive control adiponectin (250 ng/mL). The data are presented as box plots of five to eight separate experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$ (Mann-Whitney test). ((a)) Adiponectin, nonobese supernatant, and obese supernatant vs. control, ((b)) Obese supernatant *vs.* nonobese supernatant, ns: nonsignificant.

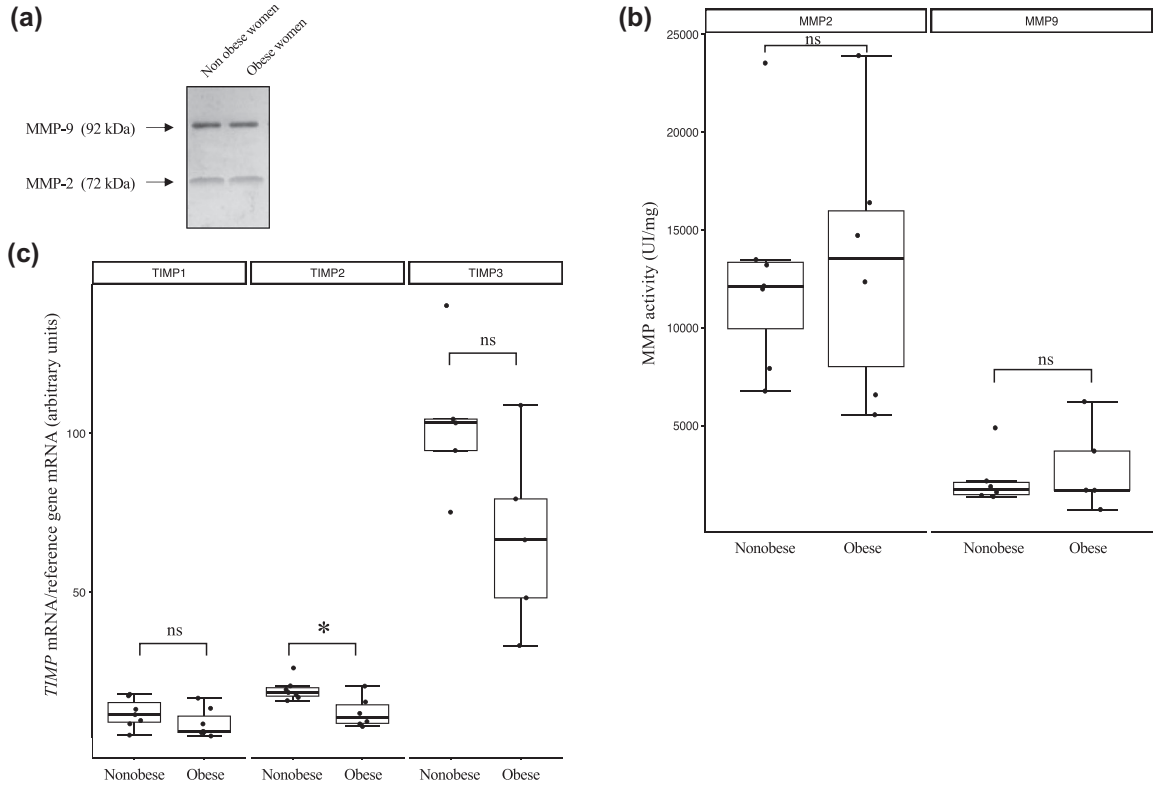

FIGURE 3 The effect of obesity on human endometrial MMP/TIMP balance. Human ESCs were cultured for 14 days in DMEM/F12 medium supplemented with E2 and P4. (a) Representative zymography of MMP2 and MMP9 activities (b) Activities of gelatinases (MMP-9 and MMP-2) in CM from decidualized ESCs isolated from nonobese women or obese women. The data are presented as box plots of 5 to 7 separate experiments. (c) Total RNA was extracted after 14 days of cell differentiation. mRNA expression levels of *TIMP-1*, *TIMP-2*, and *TIMP-3* were quantified by RT-qPCR. The data are presented as box plots of five to seven separate experiments. *$p \leq 0.05$ (Mann-Whitney test) for obese women *vs.* nonobese women, ns: nonsignificant.

3.3 | The impact of obesity on endometrial MMP activities and endometrial TIMP mRNA expression levels after 14 days of ESC culture

We next used zymography assays after 14 days of cell culture to specify the molecular mechanisms involved in the pro-invasive influence of obesity (Figure 3a). We did not observe any difference in MMP-2 and MMP-9 activities between the obese and nonobese groups (Figure 3b). In RT-qPCR assays of TIMP-1, TIMP-2, and TIMP-3 (all of which are strongly expressed in human endometrium), we observed significant lower TIMP-2 mRNA expression (a relative decrease of 37%; $p < 0.05$) in ESCs isolated from obese women than in ESCs isolated from nonobese women (Figure 3c).

3.4 | Isolation and characterization of endometrial EVs

We used transmission electron microscopy to characterize the morphology of EVs isolated from the endometrial biopsy supernatant *via* size exclusion chromatography. The EVs formed amorphous aggregates (Figure 4a) and ranged from 50 nm to 200 nm in size (Figure 4b). The three EV protein markers (CD9, CD81, and HSP70) were detected in an immunoblot analysis (Figure 4c). The strong expression of CD9 and CD81 confirmed that our EV preparations were enriched in ectosomes derived from plasma membranes (Kowal et al., 2016; Mathieu et al., 2021). The mean ± standard deviation size was 116 ± 6 nm for the most frequently occurring endometrial EVs and 144 ± 6 nm overall. The EV concentration varied from one individual preparation to another (range: 5×10^{11} to 6×10^{12} EV/ml). However, the preparations from the obese and nonobese women did not differ significantly with regard to EV concentration (Figure 4d) or size (Figure 4e).

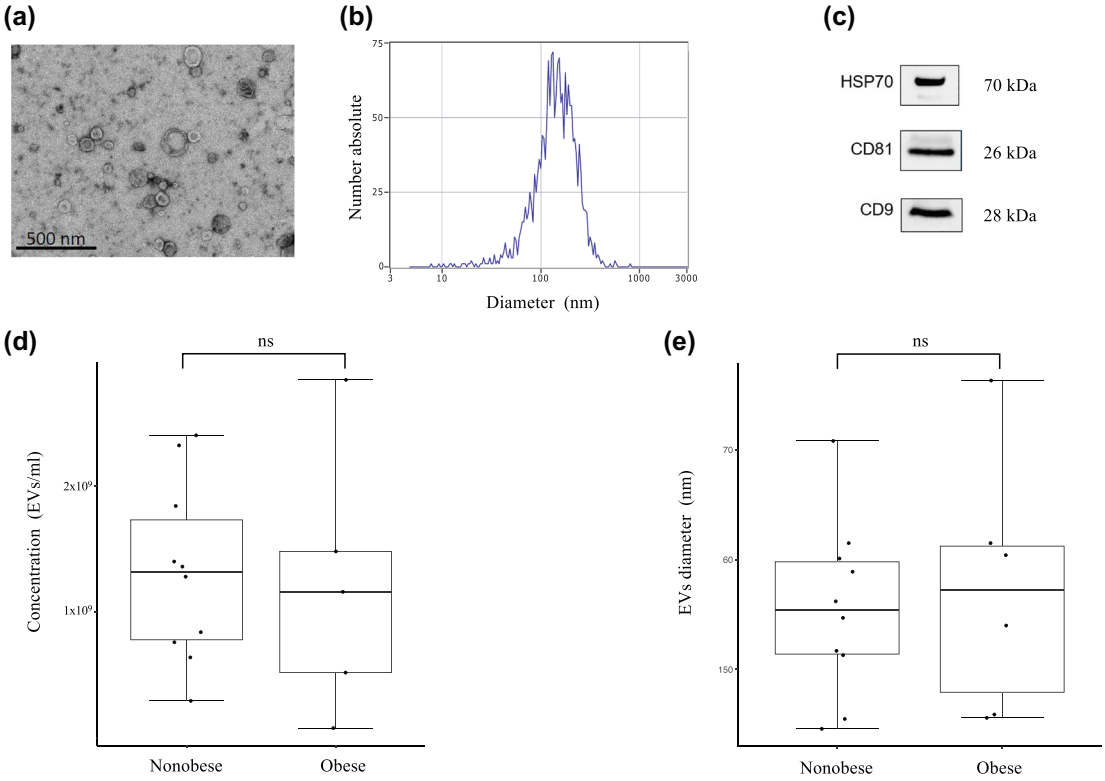

FIGURE 4 The effect of obesity on the characteristics of human endometrial EVs. EVs were isolated from human endometrial biopsies and then purified, as described in the Materials and Methods. (a) A representative transmission electron micrograph of an EV fraction. (b) A representative NTA plot of an EV fraction. (c) Specific markers of EVs identified by immunoblotting with anti-HSP-70, anti-CD81, and anti-CD9 antibodies. (d) The concentration measured by NTA (ZetaView, Particle Metrix) of endometrial EVs isolated from obese *vs*. nonobese women The data are presented as box plots of 5 obese and 10 nonobese separate EVs preparations. (e) The size distribution measured by NTA of the same endometrial EVs isolated from obese vs. nonobese women. The data are presented as box plots of 6 obese and 10 nonobese separate EVs preparations. ns: nonsignificant for obese women vs. nonobese women (Mann-Whitney test).

3.5 | The effect of EVs isolated from endometrial secretions of nonobese women on the in vitro decidualization of ESCs isolated from obese women

We performed preliminary experiments to assess the EVs' influence on in vitro decidualization. We determined whether or not the addition of EVs to the culture media (5×10^{10} EVs / 10^6 cells) for 48 h diminished the ESCs' viability. Since similar amounts of LDH were released into the supernatants of ESCs treated with EVs (6 ± 1 IU) or ESCs not treated (5 ± 2 IU), we concluded that EV supplementation did not exert any cytotoxic effect (data not shown).

Once these prerequisites had been checked, we found that treatment (a pulse at day (D)7-9 of culture) with pools of EVs isolated from 5 nonobese biopsy supernatants corrected the defective in vitro decidualization observed in ESCs from obese women, as reflected by PRL secretion and *PRL* mRNA expression after 14 days of culture (Figure 5). The level of PRL secretion was restored to that seen after 14 days of differentiation of ESCs from nonobese women (Figure 1).

3.6 | The effect of EVs isolated from endometrial secretions of obese and nonobese women on trophoblastic cells' invasive abilities in vitro

In order to determine the EVs' putative involvement in the endometrial control of trophoblast invasion, we performed a series of Matrigel® Transwell® invasion assays. The HTR-8/SVneo immortalized EVT cell line was cultured (or not) with EVs isolated from endometrial secretions of obese or nonobese women. As shown in Figure 6, we found that treatment of HTR-8/SVneo cells with EVs from obese women was associated with 30% more invasive activity ($p < 0.05$), relative to EVs from nonobese women.

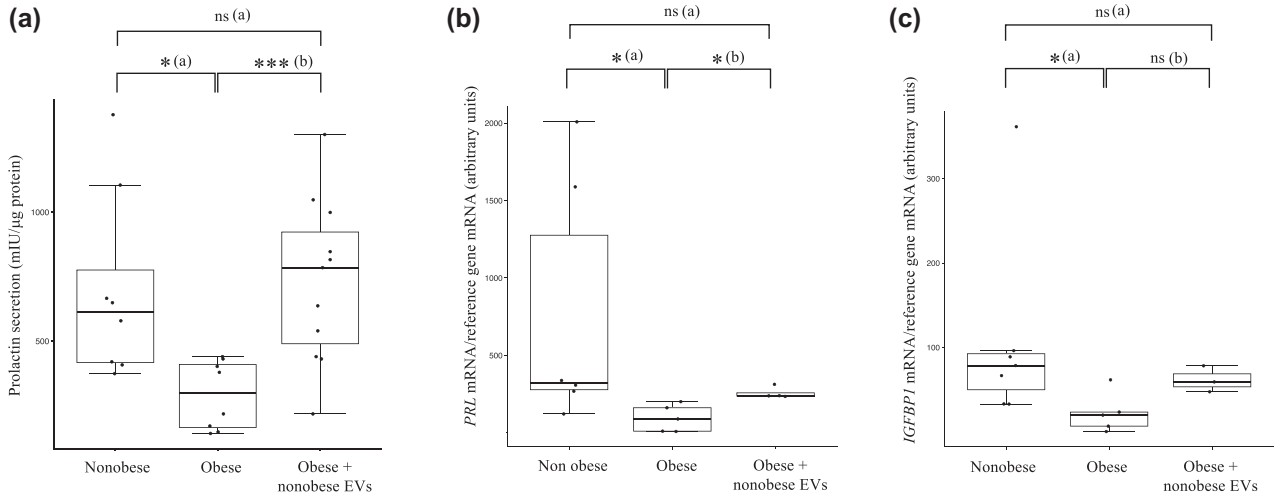

FIGURE 5 The effect of EV supplementation on the in vitro decidualization of ESCs. After 7 days of culture in differentiation medium (DMEM/F12 supplemented with E2 (10−8 M) and P4 (10−6 M)), ESCs isolated from nonobese women or obese women were treated (or not) for 48 h with EVs isolated from endometrial secretions of nonobese women. The cells were then incubated with differentiation medium. (a) Prolactin secretion into the endometrial supernatants was measured on D14 of cell differentiation in the presence or absence of a pulse of EVs at D7. The data are presented as box plots of 8 to 11 separate experiments. (b-c) Total RNA was extracted on D14. mRNA expression levels of PRL (b) and IGFBP-1 (c) were quantified in RT-qPCR assays. The data are presented as box plots of 3 to 7 separate experiments. *$p \leq 0.05$, ***$p \leq 0.001$ (Mann-Whitney test). ((a)) Obese, obese + nonobese EVs vs. nonobese. ((b)) Obese + nonobese EVs vs. obese, ns: nonsignificant.

TABLE 2 The 16 differentially expressed proteins (EVs from obese women vs. EVs from nonobese women), according to XIC analysis.

Accession N°	HUGO	Full name	NOB/OB	*p*-value	p-adjust (BH)
O95497	VNN1	Vanin1	3.0	0.0005	0.0510
P15144	AMPN	Aminopeptidase n	1.6	0.0007	0.0510
Q6IAA8	LTOR1	Ragulator complex protein	1.8	0.0018	0.0833
Q86VB7	CI63A	Scavenger receptor cysteine-rich type 1 protein m130	2.2	0.0025	0.0833
P00488	F13A	Coagulation factor xiii, a1 subunit	2.7	0.0027	0.0833
Q14254	FLOT2	Flotillin 2	1.8	0.0038	0.0833
P04275	VWF	von Willebrand factor	2.9	0.0041	0.0833
P27797	CALR	Calreticulin	0.6	0.0043	0.0833
P50895	BCAM	Basal cell adhesion molecule	1.5	0.0054	0.0908
P67809	YBOX1	Y-box binding protein 1	0.6	0.0059	0.0908
O43301	HS12A	Heat shock 70	1.6	0.0071	0.0908
Q15046	SYK	Lysine-tRNA ligase	0.5	0.0075	0.0908
P60903	S100A10	Protein s100-a10	2	0.0087	0.0908
P02751	FINC	Fibronectin	4.54	0.0089	0.0908
P12821	ACE	Angiotensin-I-converting enzyme	1.6	0.0092	0.0908
O43707	ACTN4	Alpha-actin-4	1.5	0.0094	0.0908

3.7 | The protein content of EVs isolated from human endometrial secretions

In view of the effect of EVs on ESC cultures, we used quantitative mass spectrometry analyses to define and compare protein compositions and the associated biological processes in EVs isolated from human endometrial secretions during the implantation window in obese versus nonobese women. Our proteomic analysis of obese versus nonobese samples highlighted the presence of a large number of differentially expressed secreted and cellular proteins (100 from the SC analysis (Supplementary Table I) and 16 from the XIC analysis (Table 2) and produced a detailed list of proteins that were common to the two analysis or were restricted to the XIC analysis (Figure 7a). In terms of molecular functions, most of the differentially expressed proteins were involved in

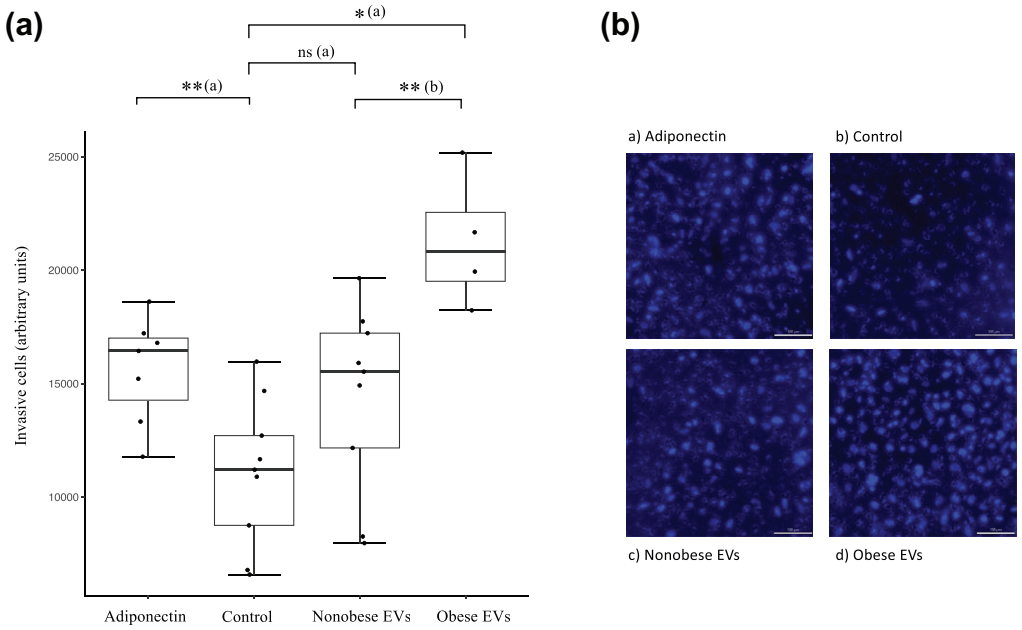

FIGURE 6 Direct effects of human endometrial EVs on trophoblast invasion. (a) HTR-8/SVneo cells were cultured for 48 h in the presence of control medium supplemented (or not) with the positive control adiponectin (250 ng/mL) or supplemented with purified EVs isolated from endometrial secretions of nonobese or obese women. The data are presented as box plots of four to nine separate experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ (Mann-Whitney test). ((a)) Adiponectin, nonobese EVs, and obese EVs *vs*. control, ((b)) Obese EVs *vs*. nonobese EVs, ns: nonsignificant. (b) Representative microphotographs from nine separate experiments, showing fixed, Hoechst-stained HTR-8/SVneo cells on the bottom side of the Transwell® membrane (magnification: ×10). HTR-8/SVneo cells were cultured for 48 h in the presence of adiponectin ((a)), in the absence of adiponectin (control) ((b)), with EVs from nonobese women ((c)), or with EVs from obese women ((d)).

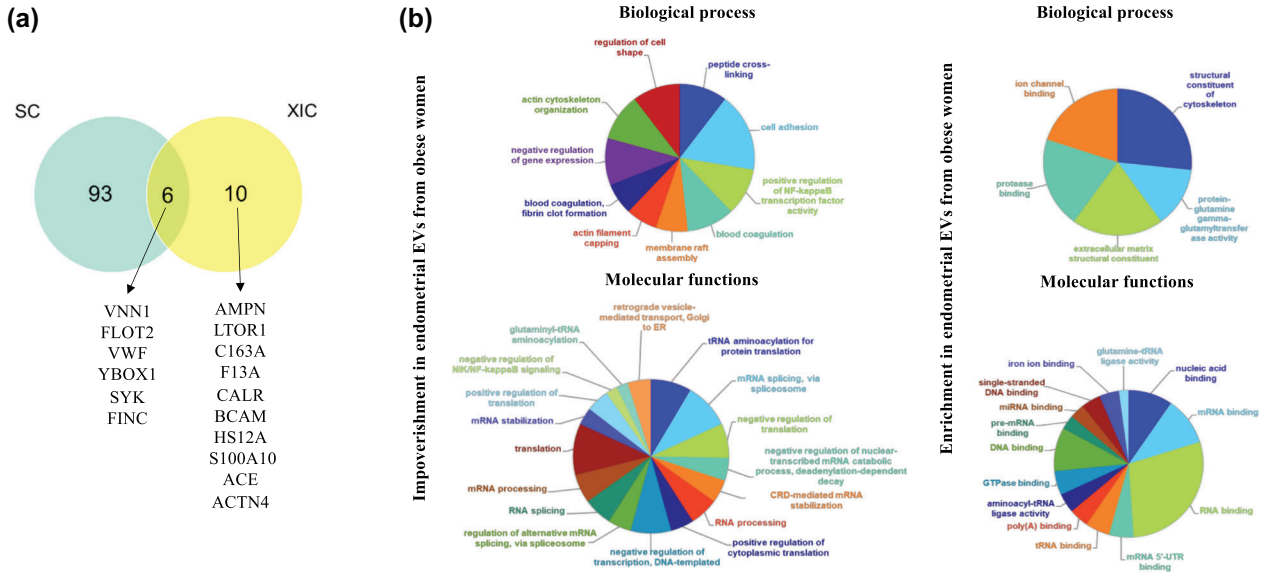

FIGURE 7 The effect of obesity on human endometrial EV content. Endometrial EV content was studied using proteomic techniques. (a) A Venn diagram showing the proteins identified respectively in the SC and the XIC analysis, together with the detailed list of proteins common to the two analyses or restricted to the XIC analysis. (b) The biological processes and molecular functions identified in functional enrichment analyses, using the DAVID databases.

protein translation and mRNA splicing. In terms of biological processes, most were involved in cell remodelling, endocytosis, and angiogenesis (Figure 7b).

Our proteomic data revealed as well the presence of well-known EVs markers like Alix (PDCD6IP), HSP70, CD81, CD9, and also CD63 (but at low level) (Kowal et al., 2016; Mathieu et al., 2021). Moreover, our proteomic results suggested that the EVs produced by endometrial biopsies were comparable to endometrial EVs described in the literature. Indeed, our results demonstrated that EVs contained many proteins that have been revealed by proteomics studies performed on whole endometrial tissue

containing both stromal and epithelial cells, or after laser microdissection in human (Evans et al., 2020) and in cow (Chankeaw et al., 2021). We observed, for instance, the presence of several proteins implicated in receptivity and implantation processes: (i) fibronectin [FINC, (Kayisli et al., 2005)] and serpins [SERPH, A1AT, (Fitzgerald et al., 2023)] as stromal cell markers; (ii) podocalyxin [PODXL, (Paule et al., 2021)], mucin [MUC1, (Margarit et al., 2010)], and claudin [CLD3, (Schumann et al., 2015)] as epithelial cell markers, and iii) S100A10 which is expressed in both stromal and epithelial cells (Bissonnette et al., 2016). Finally, our EVs also contained several proteins like: C3 complement, annexin (ANX), Filamin A (FLNA), alpha actinin (ACTN4), actin cytoplasmic-1 et 2 (ACTB, ACTG), Alix (PDCD6IP), HSP90 A et B, and HSPA5 (BIP), which were previously described in either in vivo EVs isolated from uterine lavages (Rai et al., 2021), or EVs produced by ESCs after in vitro decidualization (Gurung et al., 2021). As a result, our data suggest that EVs produced by endometrial biopsies, in our experimental conditions, are representative of endometrial EVs secreted by ESCs either in vitro or in vivo.

Since our study's objective was to characterize the EVs' involvement in the regulation of both decidualization and endometrial control of trophoblast invasion (both of which are essential for successful implantation), we next performed a more detailed analysis of proteomic experiments. With regard to decidualization, we demonstrated that only one protein (aminopeptidase-A (AMPE)) was more abundant in endometrial EVs from obese women than in endometrial EVs from nonobese women (by a factor of 0.6, in the SC analysis). Aminopeptidase-A is known to downregulate decidualization (Mizutani et al., 2020). Conversely, five proteins were less abundant in endometrial EVs from obese women than in endometrial EVs from nonobese women: S100A10 (less abundant by a factor of 2 in the XIC analysis) is the heterodimeric partner of annexin-2 and has a crucial role in the acquisition of the endometrial receptivity phenotype (Bissonnette et al., 2016); ankyrin 1 (ANK1) (less abundant by a factor of 1.7 in SC) contributes to the endometrium's adhesive capacity and thus embryo attachment (Zafir et al., 2021); aminopeptidase N (AMPN, less abundant by a factor of 1.6 in the XIC analysis) is released into the uterine cavity *via* the shedding of microvesicles from endometrial epithelial cells (Shui et al., 2019; Yoshihara et al., 2021); fibronectin (FINC, less abundant by a factor of 4.5 in the XIC analysis) can induce decidual cell differentiation itself after binding to aminopeptidase N (Kayisli et al., 2005; Yoshihara et al., 2021); and angiotensin-converting enzyme (ACE, less abundant by a factor of 1.6 in the XIC analysis) involved in angiogenesis *via* the activation of endometrial pro-renin receptor/renin angiotensin (Lumbers et al., 2015). With regard to the invasive phenotype, our proteomic results showed that over 100 proteins were differentially expressed in obese women *vs*. nonobese women and appeared to be involved in invasive processes in general. Only two of these proteins were already known to be involved specifically in trophoblast invasion. We demonstrated that basal cell adhesion molecule (BCAM, involved in trophoblast proliferation, migration, and invasion; Liu et al., 2022) was significantly less abundant in endometrial EVs from obese women than in endometrial EVs from nonobese women (by a factor of 1.6 in the XIC analysis). Conversely, we found that cofilin-1 (COF1, which is involved in actin remodelling and dynamics; Ali et al., 2019) is significantly more abundant in endometrial EVs from obese women than in endometrial EVs from nonobese women (by a factor of 0.6 in the SC analysis). Since these seven proteins are differentially expressed in endometrial EVs from obese *vs*. nonobese women, they might be potential biomarkers of endometrial receptivity.

4 | DISCUSSION

A growing body of evidence from epidemiologic and animal studies shows that obesity alters endometrial receptivity, which in turn leads to subfertility (Bellver et al., 2013; Provost et al., 2016). However, the underlying cellular and molecular mechanisms have not yet been identified or characterized.

The present study is the first to demonstrate that obesity reduces the biochemical differentiation of ESCs, as evidenced by the significantly lower levels of PRL and IGFBP-1 expression in ESCs isolated from obese women (relative to ESCs from nonobese women). These results are in line with literature reports in which excess adiposity in humans perturbed the uterine environment, with local inflammation, immune system changes, and mitochondrial stress (Bellver et al., 2011; Comstock et al., 2017). Various data show that fully differentiated ESCs (e.g., decidual cells) regulate the MMP/TIMP balance and thus influence the human trophoblast's pro-invasive properties (Sharma et al., 1989). We therefore sought to determine whether obesity also affects the ECSs' ability to modulate the trophoblast invasion process. Our results showed clearly that exposure of trophoblastic cells to CM from ESCs isolated from obese women and cultured for two weeks was associated with significantly greater trophoblast invasion activity. Furthermore, our results showed that this accentuation of trophoblast invasion by ESCs from obese women was significantly and specifically associated with a low level of TIMP-2 mRNA expression by human ESCs. Thus, excess body fat might enhance the endometrial control of trophoblast invasion. Our present results extend the findings of earlier studies in which early trophoblast invasion was observed in women with various reproductive disorders (including PCOS, pre-eclampsia, and recurrent pregnancy loss) (Palomba et al., 2014; Schatz et al., 2016). In contrast, Antoniotti et al. reported that "obese" levels of advanced glycation end products (which have pro-inflammatory actions) were associated with less invasive activity, relative to "lean" levels of these products (Antoniotti et al., 2018). There are several possible explanations for these discrepancies. Firstly, Antoniotti et al. focused on advanced glycation end products in uterine secretions, whereas we studied the totality of endometrial secretions including EVs. Secondly, we applied a large number of exclusion criteria (PCOS, endometriosis, recurrent pregnancy

loss, uterine anomalies, endocrine dysregulation, cigarette smoking, etc.) to our two groups of participants, in order to recruit well-matched populations and minimize bias in the results. It is well known that nicotine's anti-estrogenic activity significantly influences placental function since it significantly reduces trophoblast invasion (Raez-Villanueva et al., 2018). Hence, the discrepancies between these two studies might result (at least in part) from anthropometric, hormonal, metabolic, immunologic, and environmental differences.

It is becoming increasingly clear that EVs have critical roles in the regulation of decidualization (Paule et al., 2021; Rodriguez-Caro et al., 2019) and trophoblast invasion (Liu et al., 2020; Rai et al., 2021) in humans. In the present study, we investigated the potential involvement of endometrial EVs in the impairment of uterine receptivity described in obese women. We first compared obese and nonobese women with regard to the qualitative and quantitative characteristics of their endometrial EVs but did not find any significant intergroup differences; the purified EVs had the same size distributions, morphologies and concentrations.

Furthermore, we performed various functional assays, with a view to determining the putative involvement of endometrial EVs in the weaker in vitro decidualization and enhanced endometrial control of trophoblast invasion in obese women. To study the decidualization process in vitro, we used a D7 pulse of endometrial EVs purified from nonobese women. Day 7 was chosen because a recent study of a human endometrial cell line found that in the presence of 1 μM of P4, the in vitro morphological differentiation rate peaked at D7-9 and PRL secretion peaked on day 7 (Suthaporn et al., 2021). Moreover, this timepoint is consistent with the physiological cycle: since ovulation typically occurs on day 14 of the menstrual cycle (in women with 28-day cycle), D7-9 after ovulation corresponds to days 21–23 – the putative implantation window (Harper, 1992). Our results demonstrated that in vitro supplementation with EVs isolated from nonobese subjects corrected the defect in ESC differentiation (as evidenced by PRL secretion) observed in obese women. To study the trophoblast invasion process, we compared the invasive properties of HTR-8/SVneo immortalized EVT cell line after two days of exposure to endometrial EVs purified from obese *vs*. nonobese women. Our results demonstrated that trophoblast invasion was significantly greater with the "obese" EV preparation. Our two different experimental procedures (using CM from fully decidualized ESCs, and purified EVs from endometrial supernatants) showed the same range of trophoblast invasive stimulation in samples from obese women suggesting that this pro-invasive action is conveyed (at least in part) by the EV fraction.

In view of the results of our mass-spectrometry-based quantitative proteomic analysis (as detailed in the Results section) and our functional assays, we hypothesize that (i) fibronectin and angiotensin converting enzyme are involved in the recovery of decidualization capacity of ESCs from obese women (since these proteins are abundantly present in endometrial EVs from nonobese women and are both involved in decidual processes; Kayisli et al., 2005; Lumbers et al., 2015) and (ii) cofilin-1 is involved in controlling the trophoblast's invasive abilities in the context of obesity (since this protein is abundantly present in endometrial EVs from obese women and exerts a pro-invasive action; Ali et al., 2019). Thus, the results of the present study suggest that endometrial EVs transport cargo proteins could regulate both decidualization and trophoblast invasion processes. Experiments to test these hypotheses are underway in our laboratory.

In conclusion, the present study is the first to have demonstrated that treatment with endometrial EVs from nonobese women fully restored the decidualization defect observed in vitro in obese women. These encouraging results strengthen the hypothesis whereby EVs constitute a novel therapeutic strategy in this field. Although EV therapy might become a promising method for repairing damaged endometria, this novel approach has some ethical, biological and technical limitations (e.g., collection difficulties and immunogenicity). However, EVs can be easily isolated from endometrial secretions and can carry biologically active molecules. Our results indicate that the use of endometrial EVs might constitute a novel cell-free therapeutic strategy for promoting endometrial function and (perhaps) increasing the likelihood of success for obese women in in vitro fertilization programs. Indeed, EVs' potential for protecting tissues against environmental and physiological stressors has already been evidenced in various oxidative-stress-associated pathological contexts, such as PCOS, and endometritis (Tesfaye et al., 2021).

AUTHOR CONTRIBUTION

Laurent Galio: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review & editing. **Laetitia Bernet**: Data curation; formal analysis. **Yoann Rodriguez**: Data curation; formal analysis. **Camille Fourcault**: Data curation; formal analysis. **Marie-Noëlle Dieudonné**: Conceptualization; fundingacquisition. **Hélène Pinatel**: Data curation; formal analysis. **Céline Henry**: Data curation; formal analysis; software. **Valérie Sérazin**: Methodology; resources. **Khadija Fathallah**: Funding acquisition; methodology; resources. **Anissa Gagneux**: Methodology; resources. **Zuzana Krupova**: Methodology. **François Vialard**: Conceptualization; funding acquisition; supervision; writing-original draft; writing-review & editing. **Esther Dos Santos**: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review & editing.

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CONFLICT OF INTEREST STATEMENT

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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