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Adipocyte-Derived Extracellular Vesicles Carrying Oncogenic Proteins Drive Obesity-Associated Endometrial Cancer: Preclinical Efficacy of HO-3867 and Metformin

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Abstract

Endometrial cancer (EC) is the most common gynecologic malignancy in the United States, with obesity contributing to 57% of cases. This study delves into the molecular mechanisms of extracellular vesicle (EV) release, which carry oncogenic proteins, and examines their role in obesity-related EC. Understanding these pathways is essential for uncovering how obesity promotes EC and for developing novel preventive and therapeutic strategies. Our findings revealed a pronounced increase in EV secretion containing TMEM205, STAT5, and FAS in adipose and uterine tissues and serum from obese EC patients compared to non-cancer controls. We also detected changes in EV regulatory proteins—Rab7, Rab11, and Rab27a—in tissues and serum from obesity-associated EC cases. In a 24-week high-fat diet (HFD, 45% kcal) mouse model, we observed higher body weight, increased fat deposition, enlarged uterine horns, and greater inflammation in HFD-fed mice. These changes correlated with elevated EV release, increased levels of TMEM205, FAS, and STAT5, and reduced PIAS3 expression in adipose and uterine tissues. Additionally, adipocyte-derived EV enhanced EC cell proliferation, migration, and tumor growth in xenografts. Treatment with HO-3867 or Metformin decreased EV release in vitro and in vivo, suppressed high glucose- or adipocyte-stimulated EC cell proliferation, and reduced body weight and fat accumulation in HFD mice. These treatments also prevented HFD-induced hyperplasia by modulating EV-regulated proteins and lowering oncogenic protein expression. Overall, this work provides mechanistic insight into obesity-driven EV release with oncogenic cargo in EC and supports further exploration of EV-targeted strategies to prevent obesity-mediated EC.

Keywords: Cancer, Ovarian cancer, Endometrial cancer, Metformin

Introduction

Endometrial cancer (EC) is the leading gynecologic cancer in developed countries and ranks sixth in incidence among women globally [1, 2]. The recent rise in cases is closely linked to the obesity epidemic. Meta-analytic data indicate that for every 5 kg/m² increase in BMI, EC risk increases significantly (risk ratio [RR] 1.59, 95% confidence interval [CI] 1.59–1.68), particularly for type 1 EC. Women with class III obesity (BMI ≥ 40 kg/m²) have a lifetime EC risk of 10–15%, compared to 2% in the general population [3]. Obesity also worsens cancer outcomes, doubling the risk of mortality from EC [4]. These observations underscore the need to study obesity-related biomarkers for cancer detection and intervention.

Obesity promotes EC in both pre- and post-menopausal women via several mechanisms. Excess adipose tissue increases circulating estrogens through aromatase-mediated androgen conversion, stimulating endometrial growth and gene transcription [5]. This effect is most

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pronounced when progesterone is low, such as in post-menopausal or anovulatory states. Visceral fat also causes chronic inflammation, contributing to hyperinsulinemia, hyperglycemia, and reduced anti-inflammatory cytokines. Altered adipose tissue function affects the secretion of adipokines and extracellular vesicles (EV) [6, 7], which may carry oncogenic proteins increasing cancer risk [8, 9].

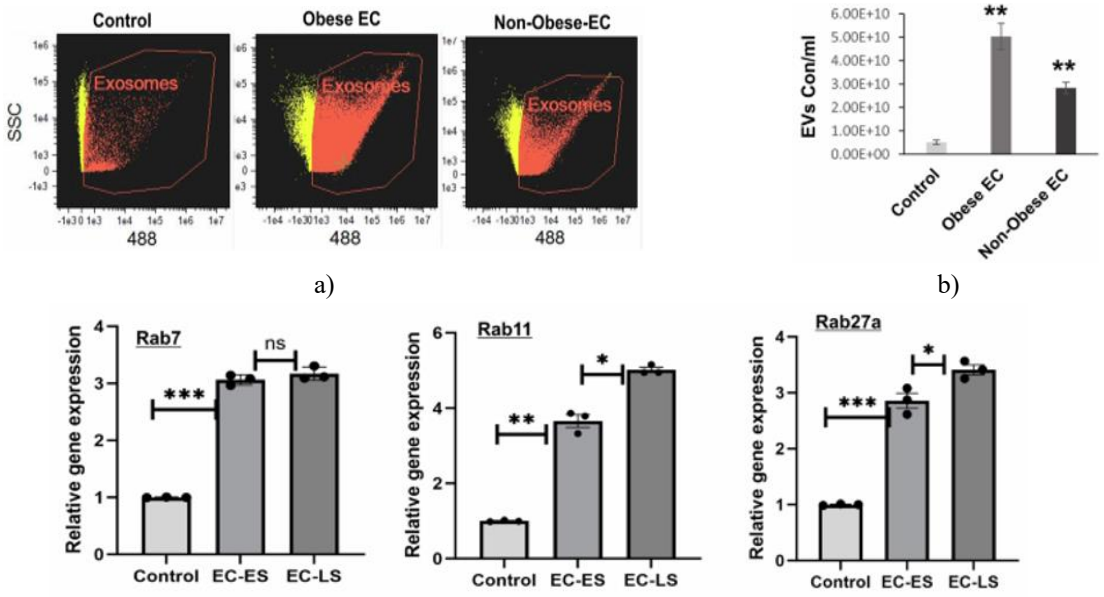
EVs are nano-sized vesicles (30–120 nm) released by various cells, with adipose-derived EVs mediating key signaling between adipocytes and neighboring cells [10–12]. The role of obesity-driven EV signaling in the transition from atypical hyperplasia to EC remains poorly understood. Our study investigated EV secretion and oncogenic proteins (TMEM205, STAT5, FAS) in obesity-associated EC tissues and serum. TCGA data and prior studies indicate these proteins are overexpressed in EC [13, 14]. Understanding these molecular pathways is critical for developing prevention strategies, early detection biomarkers, and targeted therapies, including for drug-resistant EC.

We have developed multiple small-molecule inhibitors for ovarian and EC. Here, we employed diarylidenyl-piperidone (DAP-HO-3867) to target EV release and oncogenic proteins. HO-3867 contains an N-hydroxypyrroline (-NOH) moiety, convertible to a nitroxide, which provides selective cytotoxicity against cancer cells while sparing normal tissues [15, 16]. This selective effect allows effective inhibition of STAT3 and FAS, key drivers in our study [16, 17]. Both HO-3867 and Metformin showed efficacy in HFD-induced EC models and cell cultures, suggesting that targeting EV pathways could offer a safe and focused therapeutic approach for obesity-associated EC.

Results and Discussion

EV secretion and regulatory proteins in obesity-related EC patient samples

EVs are recognized as critical mediators of tumor progression and metastasis [18–20]. We investigated EV secretion, quantified levels, and identified regulatory proteins in obesity-associated EC patients. Using Image Stream Flow Cytometry Analysis (ISA), we detected a significant increase in EVs in serum from obese EC patients compared with non-obese EC patients and healthy controls (**Figures 1a and 1b**). Analysis of EV regulatory proteins showed upregulation of Rab7, Rab11, and Rab27a in early-stage (EC-ES) and late-stage (EC-LS) obese EC tissues compared with obese benign tissue (**Figure 1c**). We also observed higher levels of oncogenic proteins (TMEM205, STAT5, FAS) and downregulation of tumor suppressor PIAS3 (**Figure 1d**). Serum EV from obese EC patients was enriched in TMEM205, STAT5, and FAS compared with controls (**Figure 1e)**. These data suggest that elevated EVs and their protein cargo play a central role in obesity-associated EC and highlight potential serum EV biomarkers for early detection and therapeutic targeting.



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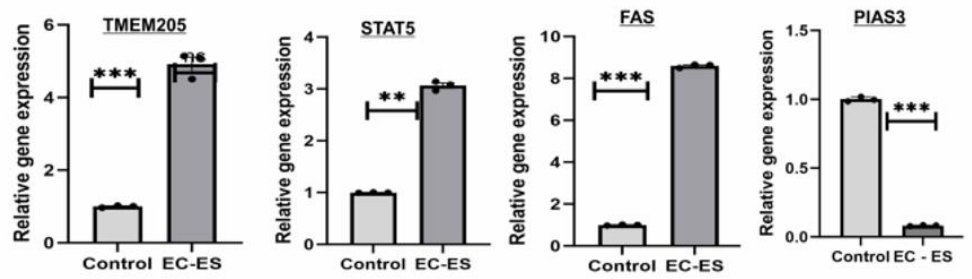

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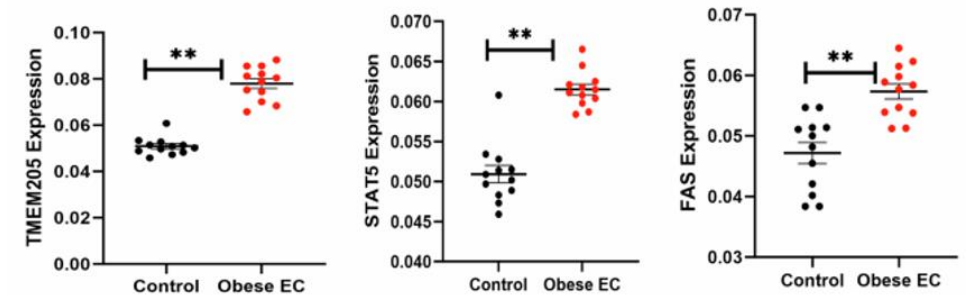

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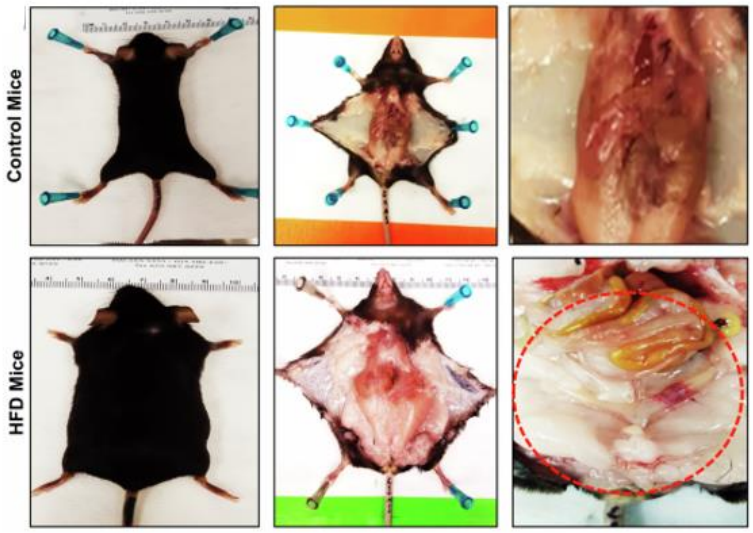
Figure 1. Heightened release of extracellular vesicles (EVs) connected to cancer-promoting proteins and proteins that control EV processes in specimens from individuals with endometrial cancer (EC).

A, B Blood serum from healthy individuals (cancer-free), those with obesity and EC, and those with EC but without obesity was melted on ice and diluted (1:4 to 1:10) using 1× PBS. EVs were separated from 100 µL serum volumes with the q-izon column method. The quantity of EVs was measured through Image Stream Flow Cytometry (ISF), which indicated a notable rise in both EV numbers and relative increase in cases of obese EC versus non-obese EC ($n = 5$, $p < 0.01$) [1]. C Proteins involved in EV control (Rab7, Rab11, and Rab27a) had their expression checked via RT-PCR across tissue from obese benign cases, early-stage obese EC (EC-ES), and late-stage obese EC (EC-LS) ($n = 3$, $p < 0.001$ or 0.005) [1]. D Levels of gene expression for cancer-related proteins (TMEM205, STAT5, FAS, and PIAS3) were tested using RT-PCR in tissues from obese benign controls and early-stage EC patients ($n = 3$, $p < 0.001$ or 0.005) [1]. E Increased protein amounts of TMEM205, STAT5, and FAS were detected in EVs isolated from serum of obese EC cases in comparison to cancer-free controls, as measured by ELISA ($n = 12$, $p < 0.005$) [1]. Assessment of how prolonged high-fat diet (HFD) intake drives endometrial hyperplasia by boosting EV production and changing expression of specific proteins To explore the consequences of ongoing consumption of a high-fat diet (HFD) (45 kcal% from fat) on EV release and the control of cancer-associated proteins within fat and uterine tissues, the investigation started with tracking body mass and uterine structure in mice subjected to 24 weeks of HFD feeding. Predictably, animals receiving HFD demonstrated much greater body mass and more fat tissue buildup than those on normal chow (**Figures 2a–c**) [2]. Strikingly, the HFD animals showed evident expansion of the uterine horns (suggesting hyperplasia) (**Figures 2d**) [2], together with clear signs of inflammation and elevated cell growth in the endometrium (**Figure 2f**) [2].



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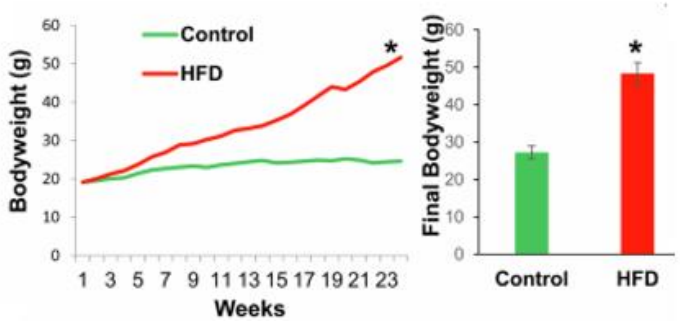

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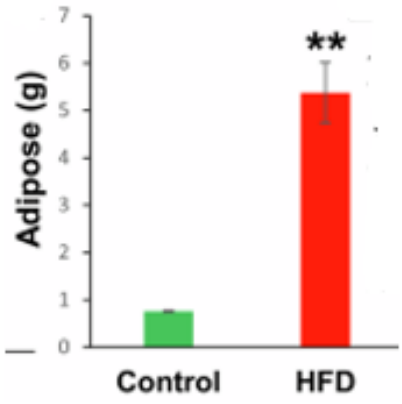

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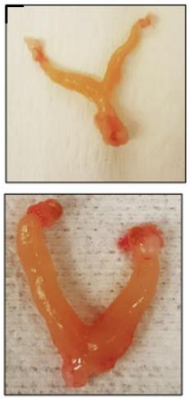

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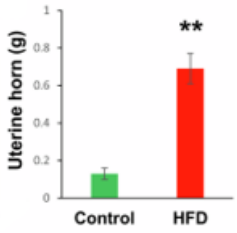

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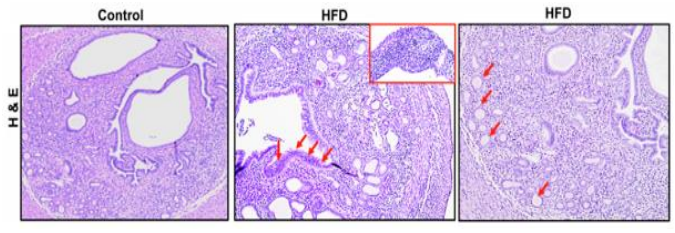

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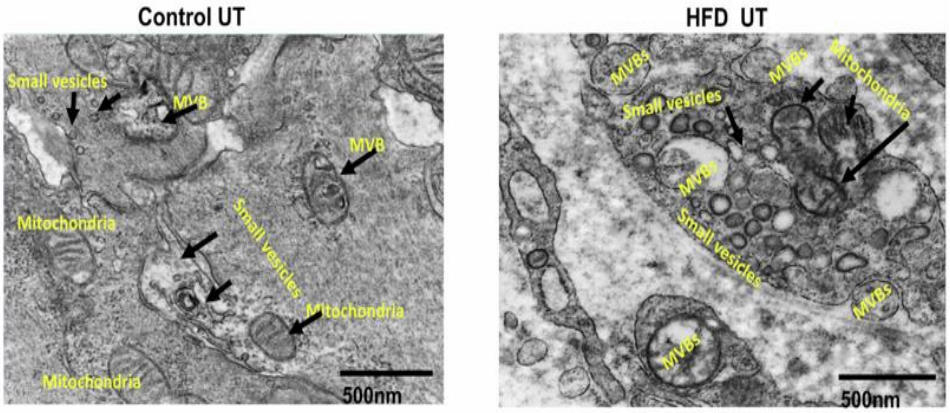
Figure 2. HFD enhances fat accumulation and drives endometrial hyperplasia.

A, B Immunocompetent mice were subjected to a high-fat diet (45% kcal from fat) for 24 weeks. Representative images indicate areas of increased adipose tissue (circled). Body weight was measured at baseline (8 weeks) and at 24 weeks ($n = 10$/group, $p < 0.01$). C Fat deposition was significantly higher in HFD-fed mice compared to controls ($n = 5$, $p < 0.005$). D, E Both uterine weight and horn size increased significantly in HFD-fed mice relative to controls ($n = 5$, $p < 0.005$). F H&E staining revealed elevated endometrial proliferation in HFD-fed mice; the inset highlights areas with higher cellular density.

We further investigated whether HFD influenced EV secretion in uterine tissues. At 24 weeks, uterine tissues were collected for Transmission Electron Microscopy (TEM). TEM revealed higher EV secretion and increased formation of multi-vesicular bodies in uterine tissues of HFD-fed mice **(Figure 3a).** Consistent with tissue findings, serum EV levels were elevated in HFD-fed mice (**Figures 3b and 3c**). In addition, mRNA levels of EV regulatory genes Rab11 and Rab27a were increased in both adipose and uterine tissues following HFD feeding (**Figures 3d and 3e**).



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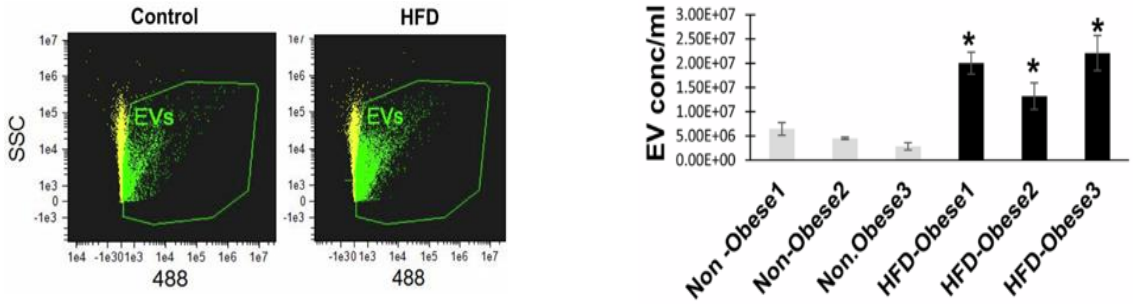

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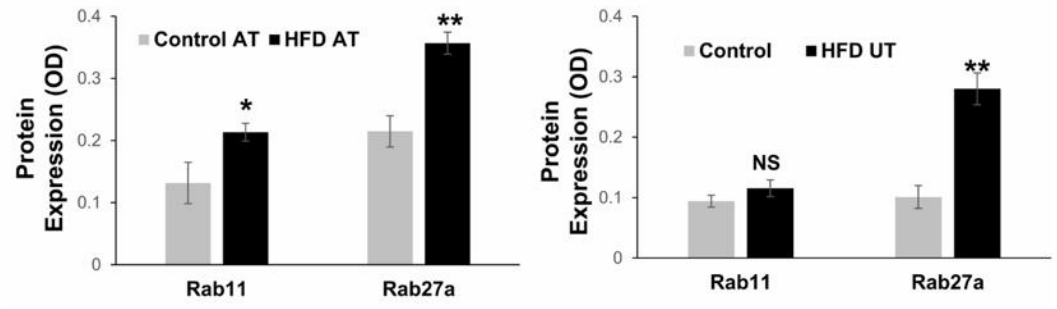

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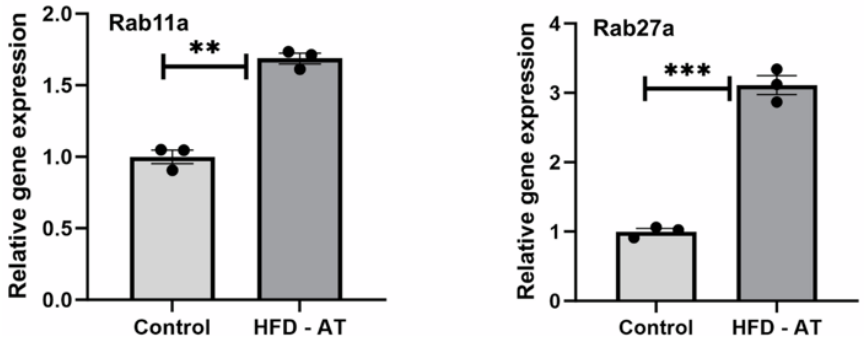

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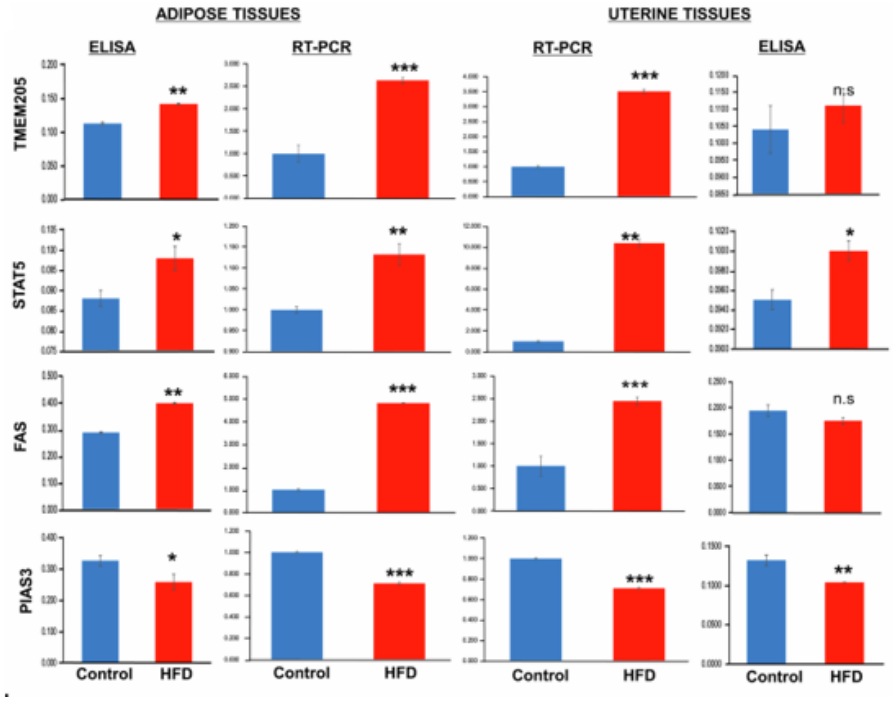
Figure 3. HFD induces EV release and modulates EV regulatory proteins in mice

A Transmission Electron Microscopy (TEM) analysis of uterine tissues revealed that high-fat diet (HFD)-fed mice exhibited a higher number of EVs and more frequent formation of multivesicular structures compared to control diet-fed mice. B, C Serum EV quantification using Image Stream technology showed a significant increase in EV abundance in HFD-fed mice relative to controls (n = 3, p*0.01).* D, E Measurement of EV regulatory molecules, Rab11 and Rab27a, in adipose and uterine tissues demonstrated elevated expression in HFD-treated mice, as determined by ELISA and RT-PCR (n = 3, p0.001 or 0.005).

In addition, HFD consumption led to notable changes in EV secretion patterns and impacted the levels of oncogenic and tumor suppressor proteins. TMEM205, FAS, and STAT5 were markedly increased, whereas PIAS3 expression was reduced in both adipose and uterine tissues (**Figures 4a and 4b**). These alterations correlate with increased cellular proliferation, endometrial hyperplasia, and early tumor formation. Overall, the data suggest that obesity-related changes in the tissue microenvironment, including elevated EV release and specific protein expression shifts, are critical drivers of EC initiation in obese patients.



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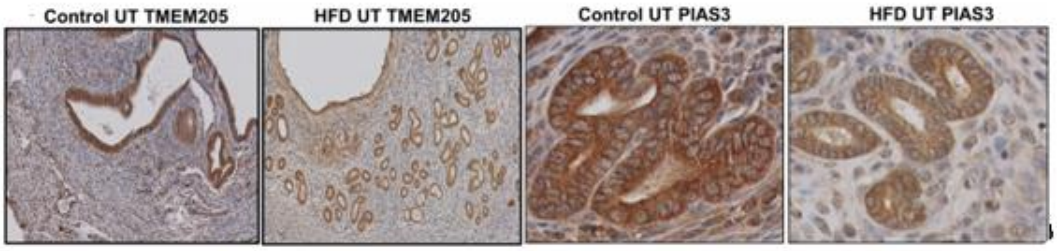

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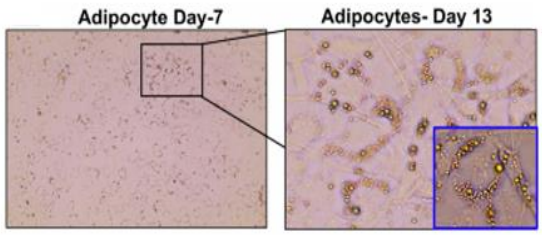
Figure 4. Changes in oncogenic and tumor suppressor proteins in HFD-fed mouse tissues.

A, B Analysis of key proteins (TMEM205, STAT5, FAS, and PIAS3) in adipose and uterine tissues from HFD- and control diet-fed mice. Gene and protein expression levels were assessed using RT-PCR, ELISA, and IHC ($n = 3$, $p < 0.01$ or 0.005).

Adipocyte-derived EVs in endometrial cancer progression

Previous research has established that adipocytes and their secreted factors play a central role in cancer development, including obesity-associated EC [19, 21–24]. In this study, we observed that extracellular vesicles from differentiated adipocytes (ADEVs) carry oncogenic proteins TMEM205, STAT5, and FAS, which can regulate EC cell behavior in vitro and in vivo (**Figures 5a–d**).

Co-culturing ADEVs with EC Ishikawa (IK) cells significantly enhanced cell proliferation (**Figures 5e and 5f**). To test in vivo effects, nude mice were xenografted with IK cells and administered ADEVs weekly near the tumor site starting two weeks post-injection. ADEV treatment led to a pronounced increase in tumor volume compared to untreated controls (**Figures 5g–i**). Protein analysis of these xenograft tumors revealed elevated TMEM205, FAS, and STAT5 levels, along with decreased Rab27 expression in the ADEV-treated group (**Figure 5j**). These results indicate that adipocyte-derived EVs promote EC progression, highlighting their role in the context of obesity-driven tumor development.



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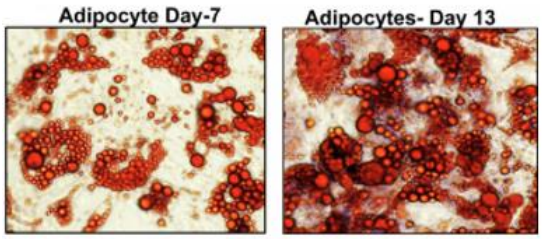

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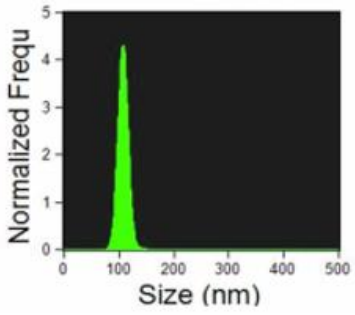

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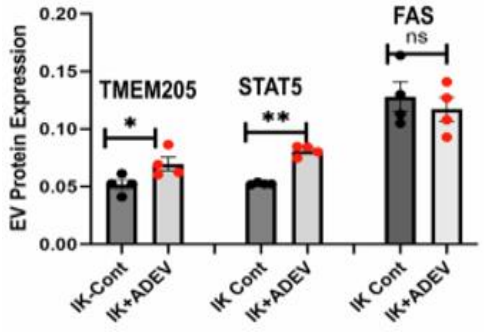

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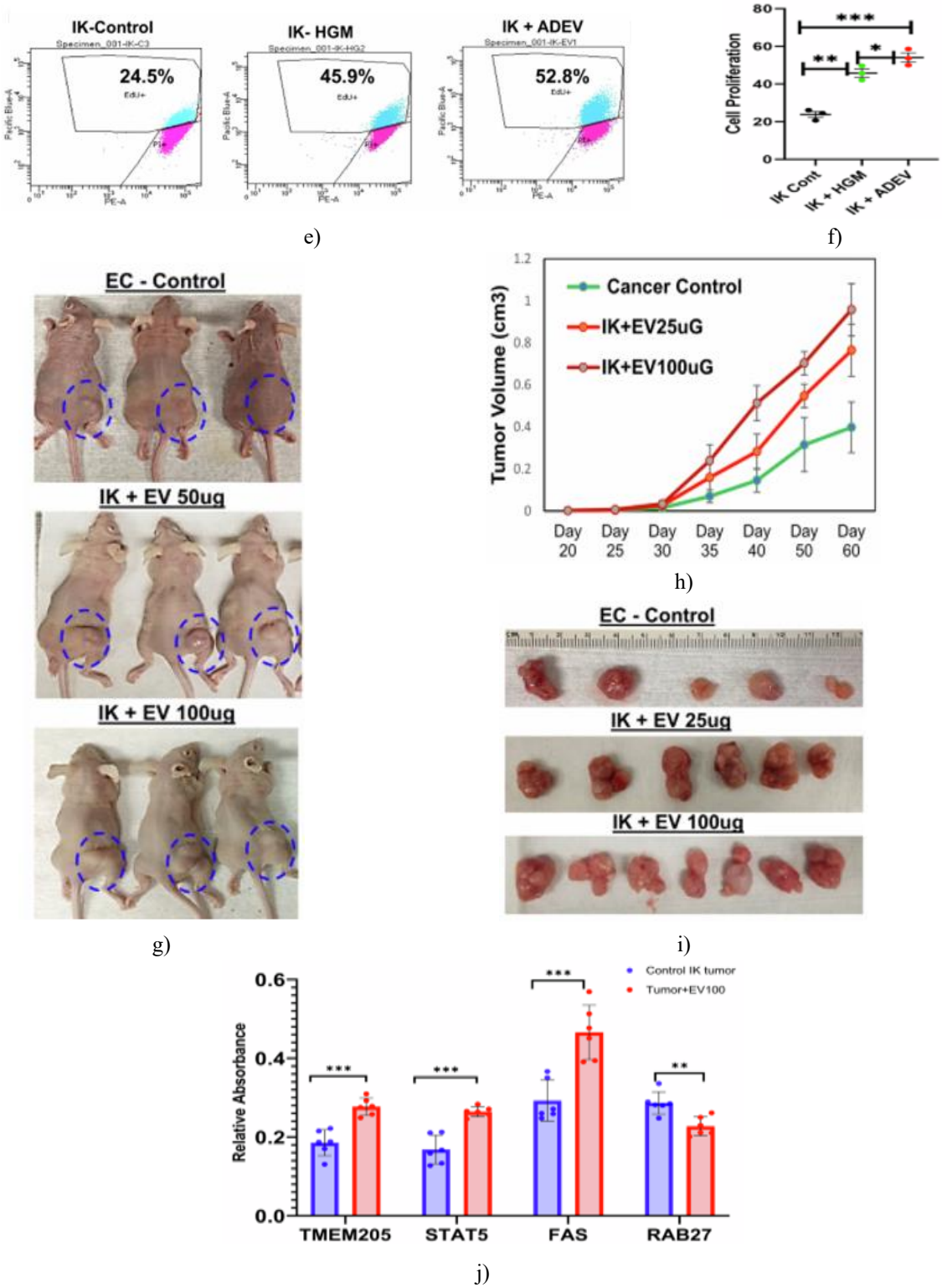

Figure 5. Adipocyte-derived EVs enhance EC cell proliferation, migration, and tumor growth**.**

A Adipose-derived mesenchymal stem cells were differentiated into adipocytes starting 72 h after reaching confluency on Days 3, 5, and 7 and maintained in adipocyte-specific medium until Day 15. Representative microscopic images (10× and 40×) illustrate progressive accumulation of lipid droplets over time. B To confirm

adipocyte differentiation, cells in 12-well plates were fixed and stained with 0.2% Oil Red O in 2-propanol for 10 min at room temperature. Lipid accumulation was quantified at different time points. C EVs secreted by adipocytes were isolated and their size and concentration were validated using Image Stream Flow Cytometry (ISF). D ADEVs were incubated with Ishikawa (IK) cells for 48 h. Protein levels of TMEM205, FAS, and STAT5 were measured by ELISA, showing significant upregulation ($n = 4$, $p < 0.01$ or 0.005). E, F Proliferation of IK cells exposed to ADEVs was assessed by EdU incorporation and flow cytometry, revealing a higher proportion of EdU-positive cells compared to untreated IK controls ($n = 3$, $p < 0.05$ or 0.01). G For in vivo studies, 2 ×10^6 IK cells were subcutaneously injected into the right flank of athymic nude mice. After one week, mice were divided into three groups: Cancer Control (IK only, $n = 6$), EV25µg (IK + 25 µg EV, $n = 7$), and EV100µg (IK + 100 µg EV, $n = 7$). ADEVs were injected subcutaneously near tumors twice weekly for 4 weeks; controls received saline. H, I Tumor size was monitored weekly using digital calipers, and tumor volumes were calculated. J Tumors were harvested at the study endpoint. ELISA analysis revealed elevated TMEM205, STAT5, and FAS, and reduced Rab27a expression in ADEV-treated tumors compared to controls ($n = 6$, $p < 0.001$).

Effect of HO-3867 and Metformin on EC cells and HFD-induced endometrial hyperplasia

Previous studies demonstrated that elevated glucose promotes EC cell proliferation and modulates oncogenic proteins and microRNAs [9]. In this study, Ishikawa cells cultured under low, normal, or high glucose for 24–72 h exhibited enhanced proliferation and migration in the high glucose condition. Treatment with EV inhibitors—Amiloride (AME), HO-3867, or Metformin—significantly reduced EV release and suppressed EC cell proliferation (**Figures and 6a–c**).

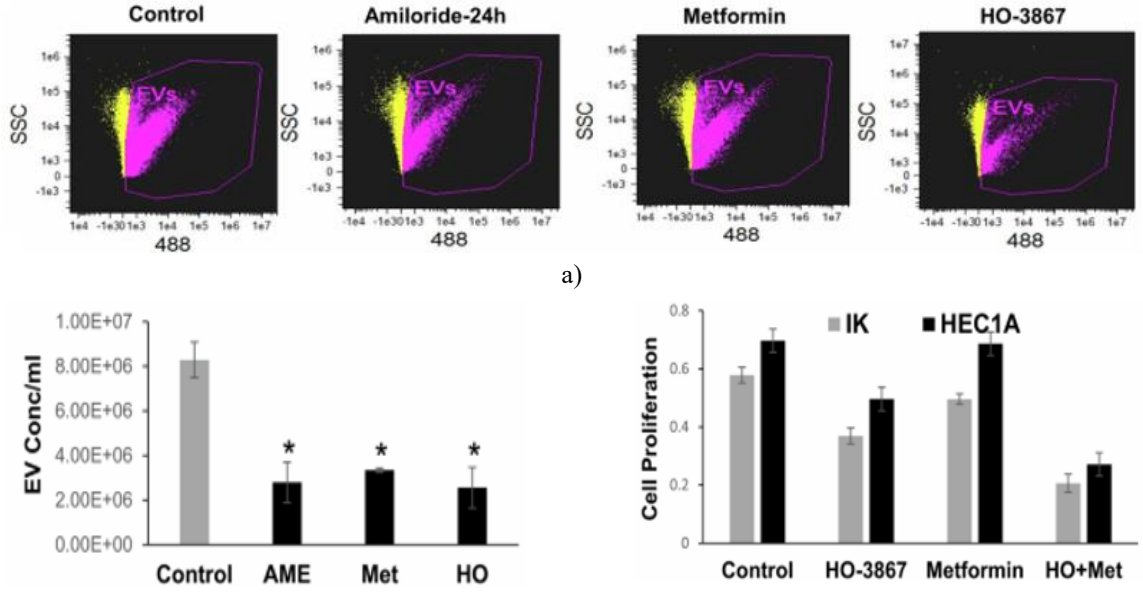

Figure 6. Influence of compounds blocking EV release on the production of EVs in endometrial cancer cells**.**

A, B Levels of EV release were examined and determined using ISF after treatment of EC cells for 24 h with the inhibitors Amiloride (10 µM), GW4869 (5 µM), Metformin (10 µM), or HO-3867 (5 µM) ($n = 3$, $p < 0.001$) [6]. C Proliferation of IK cells was evaluated following 24 h exposure to the small molecule compounds HO-3867 or Metformin ($n = 5$, $p < 0.01$) [6]. To further assess how blocking HFD-driven EV release affects the control of cancer-promoting proteins in fat and uterine tissues, the investigation utilized a small molecule compound directed at proteins in the EV control pathway (STAT3 and TMEM205) [18, 25, 26], combined with Metformin to reduce EV production. Treatment with HO-3867 and Metformin resulted in a clear tendency toward

lower body masses [7]. Notably, at the end of the study, uterine appearance, overall body mass, and fat tissue buildup in treated mice were comparable to those in untreated controls (**Figures 7a–c**) [7]. In addition, electron paramagnetic resonance (EPR) signal strength obtained from fat and uterine tissue samples of HFD-fed animals confirmed the detection of HO-3867 in its oxidized (nitroxide) state (**Figure 7d**) [7]. Tissues showing endometrial hyperplasia were analyzed by reverse transcription-polymerase chain reaction (RT-PCR), which revealed reduced mRNA levels of the selected cancer-related genes TMEM205, FAS, STAT5, c-MYC, Cyclin D2, VEGFR, and elevated levels of the cancer-suppressing gene PIAS3 in the group receiving both Metformin and HO-3867 compared to untreated animals (**Figure 7e**) [7]. Moreover, lower EV release was accompanied by diminished Rab27a mRNA expression after HO-3867 administration (**Figure 7g**) in serum and tissue specimens from mice with endometrial hyperplasia (**Figures 7f and 7g**) [7]. Overall, these findings demonstrate that HO-3867 and Metformin successfully lower the levels of cancer-promoting proteins and EV-controlling proteins, thereby inhibiting EV production in HFD-triggered endometrial hyperplasia and potentially preventing progression to EC.

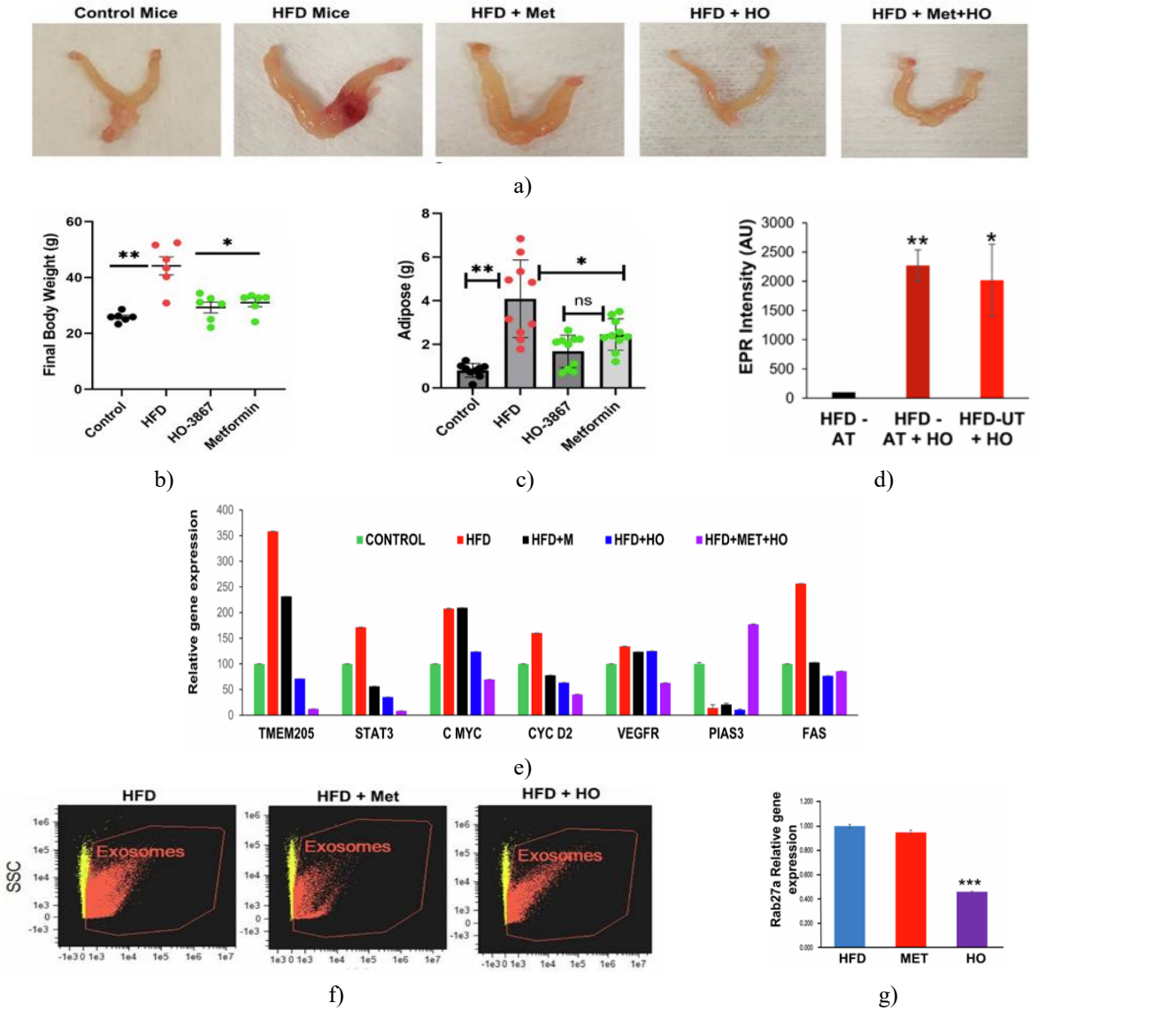

Figure7. Consequences of administering Metformin and HO-3867 on extracellular vesicle production in animals on a high-fat diet: contribution to halting endometrial cancer development.

A Example photographs displaying uterine samples from mice maintained on a high-fat diet (HFD) across 24 weeks, in comparison to specimens from those on a regular diet or given HO-3867 (2 mg/kg) or Metformin (5 mg/kg added to drinking water or feed). B, C Lowered total body mass and decreased fat deposition occurred in HFD animals upon exposure to HO-3867 or Metformin ($n = 7$ or 10, $p < 0.01$ or 0.001) [7]. D Electron paramagnetic resonance (EPR) readings taken from fat and uterine biopsies in HFD-exposed mice, verifying the occurrence of HO-3867 in oxidized (nitroxide) configuration. Amounts of HO-3867 within these samples were determined via EPR technique ($n = 4$; $p < 0.005$) [7]. E Gene expression patterns of chosen targets evaluated through RT-PCR in uterine specimens from HFD mice receiving either Metformin or the compound HO-3867 ($n = 5$, $p < 0.05$) [7]. F, G Extraction of EVs from blood serum of HFD-fed mice and those treated with DAP-HO, including assessment of EV output via Image Stream Flow Cytometry (ISF). Expression of the EV-related protein Rab27a was examined using RT-PCR in uterine samples from HFD animals administered Metformin or HO ($n = 3$; $p < 0.001$) [7].

This study provides several key findings: (i) both obese EC patients and HFD-fed mice exhibit elevated EV secretion containing oncogenic proteins in serum and tissues; (ii) oncogenes TMEM205, STAT5, and FAS are upregulated, while the tumor suppressor PIAS3 is downregulated in adipose and uterine samples from patients and HFD mice; (iii) adipocyte-derived EVs promote EC cell proliferation, migration, and disease progression; (iv) the DAP derivative HO-3867 and Metformin effectively inhibit EV release in vitro and in vivo, thereby suppressing obesity-associated EC growth. Although obesity's link to endometrial hyperplasia and progression to EC is well recognized [27–29], the precise cellular mechanisms remain incompletely defined. Proposed pathways include chronic inflammation due to altered adipose tissue function, as well as changes in the secretion of adipokines and EVs [30–32]. Recent studies indicate that adipocytes from obese individuals secrete EVs enriched in oncogenic proteins, which may contribute to tumor initiation [8, 9, 19]. EVs from adipose tissue facilitate intercellular communication between adipocytes and surrounding cells [33–35]. However, how obesity-driven signaling via EV secretion influences the development of atypical endometrial hyperplasia and carcinoma has remained unclear.

Our findings demonstrate that obesity-associated EVs carrying TMEM205, STAT5, and FAS critically contribute to EC pathogenesis. These EVs can fuse with neighboring cells, triggering downstream effects that suppress PIAS3 expression in both adipose and uterine tissues, promoting cellular proliferation and endometrial hyperplasia, thus initiating tumor growth. Prior studies also indicate that in multiple cancers, including in obese patients, elevated EV secretion correlates with increased oncogenic protein expression [21, 36, 37]. Collectively, our data reveal that EV-mediated signaling represents a central mechanism linking obesity to EC development and progression.

Although STAT5, FAS, and TMEM205 have established oncogenic roles in other cancers [38–40], their connection to EV secretion and obesity-related EC is not fully elucidated. Evidence suggests that STAT3 or TMEM205 co-localizes with EV regulatory proteins such as Rab8 or Rab11 [18, 41–43], particularly in ovarian cancer, where these interactions regulate EV release. Given the known roles of Rab7, Rab11, and Rab27 in vesicle trafficking [44–46], we examined whether elevated oncogenic proteins (STAT5, TMEM205, FAS) co-localize with these Rab GTPases. This co-localization may enhance vesicle recycling and boost EV secretion in obesity-associated adipose and uterine tissues, promoting EC progression. Future studies should investigate the precise molecular mechanisms by which TMEM205, STAT5, and FAS interact with Rab proteins, and how these interactions modulate Rab11 or Rab27a to increase EV secretion in obesity-driven EC.

Previous research has shown that blocking EV release using agents such as amiloride (AME) or small molecule inhibitors like GW4869 can suppress tumor growth in pancreatic, lung, and colon cancers [47–49]. However, whether interfering with proteins that regulate EV secretion or directly inhibiting EV release could prevent or treat obesity-associated endometrial cancer (EC) has not been explored. In this context, we developed a small molecule inhibitor, diarylidenylpiperidones-NOH (HO-3867). This compound selectively inhibits EV release in cancer cells without affecting normal tissues. HO-3867 contains an N-hydroxypyrroline (NOH) moiety, which functions as a nitroxide precursor and modulates cytotoxicity [16]. This structural feature provides antioxidant protection to noncancerous cells, making HO-3867 a promising and potentially safe agent to block oncogenic EV proteins such as STAT3, FAS, and

TMEM205, with potential applications for preventing EC and other obesity-associated cancers.

Although definitive evidence for the cancer-preventive or anticancer effects of HO-3867 or Metformin in EC, in vitro or in vivo, is still lacking, our findings emphasize the relevance of targeting EV secretion pathways in obesity-driven EC. In vivo studies using animal models offer the opportunity to evaluate whether HO-3867 or Metformin's inhibitory effects on EV release contribute to tumor suppression. These investigations may also reveal novel therapeutic strategies based on EV inhibition for cancer prevention.

Materials and Methods

Human samples collection and processing

Endometrial cancer patient samples (N = 20) were retrospectively obtained from The Ohio State University James Cancer Hospital and Solove Research Institute (IRB 2022C0070) and from Inova Schar Cancer Institute. Patients were clinically evaluated at the time of serum collection and classified as obese, with early or late-stage EC, prior to chemotherapy. De-identified clinical information—including age (30–70 years), BMI (Normal: 19–24.9; Overweight: 25–29.9; Obese: >30), waist circumference, EC stage (I/II, III, IV), histology, germline genetics, and disease distribution—was collected retrospectively. Control samples were selected from individuals without documented cancer, adnexal abnormalities, or other comorbidities. Serum samples were stored at −80 °C and thawed for 30–50 min prior to experimental use.

Cell culture

Endometrial cancer cell lines Ishikawa (grade 1) and HEC-1 (ATCC) were maintained in DMEM with low (1 mM), normal (5 mM), or high (25 mM) glucose concentrations. Media were supplemented with 10% heat-inactivated FBS, 2% sodium pyruvate, and 1% penicillin-streptomycin. Cells were grown to 70% confluence in 75 mm flasks at 37 °C under 5% CO2. Routine trypsinization was performed using 0.05% trypsin/EDTA, and mycoplasma contamination was monitored every two months using the ATCC® Universal Mycoplasma Detection Kit. Cells were thawed and passaged a maximum of five times before replacement with fresh frozen vials.

High-fat diet (HFD) study in mice

Female C57BL/6 mice were purchased from Charles River (MA, USA) and housed in well-ventilated cages, 2–3 per cage, with unrestricted access to food and water. Mice were divided into Normal Chow Diet (NCD, n = 10) and High-Fat Diet (HFD, n = 12) groups, age-matched. The NCD group received standard chow (3% kcal fat) until 24 weeks of age. The HFD group was placed on a diet containing 45% kcal fat (Research Diets, NJ, USA) starting at 3 months of age and maintained until 24 weeks. Mice were euthanized at 25 weeks, and tissues were collected.

For therapeutic experiments, mice were divided into five groups: NCD, HFD, HFD + HO-3867, HFD + Metformin, and HFD + HO-3867 + Metformin. Treatments were administered for 8 weeks after 16 weeks of HFD feeding. At the endpoint, uterine, ovarian, and adipose tissues were harvested for molecular analyses.

Transmission Electron Microscopy (TEM)

Uterine and adipose tissues were prepared for TEM by first dissecting and immersing them in 2.5% glutaraldehyde dissolved in 0.1 M phosphate buffer, maintained at 4 °C for at least 24 hours. Subsequently, the samples were rinsed and subjected to an additional fixation step with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes at room temperature. Post-fixation was performed using 1% osmium tetroxide, and tissues were then stained en bloc with 1% aqueous uranyl acetate. Dehydration was carried out through a graded ethanol series, followed by embedding in Eponate 12 epoxy resin (Ted Pella Inc., Redding, CA). Ultrathin sections were obtained with a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Deerfield, IL) and mounted on copper grids. Imaging was performed on an FEI Technai G2 Spirit transmission electron microscope (Thermo Fisher Scientific, Waltham, MA) using a Macrofire digital camera (Optronics, Inc., Chelmsford, MA) with AMT image capture software.

RNA extraction and RT-PCR

RNA was extracted from EC cell lines or tissue specimens using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Only RNA with an A260/A280 ratio between 1.8 and 2.1 was used for cDNA synthesis. Complementary DNA (cDNA) was generated from 1 mg of RNA using the Transcript First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA). RT-PCR was carried out with random hexamer

primers on a Veriti Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific).

EV isolation via size exclusion chromatography (SEC)

Extracellular vesicles were separated using IZON qEV size exclusion columns (Izon Science). Prior to use, columns were equilibrated to room temperature, and the 20% ethanol storage solution was flushed through, followed by 20 ml of particle-free PBS. Serum samples were diluted to a final volume of 500 µl in particle-free PBS and applied to the column, with elution performed using particle-free PBS. Eluted fractions of 500 µl each were collected, and fractions 1–4 were combined for downstream analyses such as Image Stream Flow Cytometry. For ELISA assays, pooled EV samples were concentrated using Amicon Ultra-0.5 ml centrifugal filters (10 kDa; Merck Millipore) to estimate protein content and allow relative quantification of EV-associated biomarkers across samples.

Statistical analysis

Data are expressed as mean ± S.D. Statistical comparisons between groups were performed using Student's t-test. Significance was defined as $p \leq 0.05$.

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Conflict of Interest: None

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Ethics Statement: All procedures used in this study were authorized and conducted according to the guidelines of the Ohio State University Research Institute Ethics Committee. All animal experiments were following the Animal Experimentation Ethics of the Ohio State University Animal Experimentation Research Lab, and the ethics approval number for animal experimentation was 2012A00000008-R3. The use of stored human tissues in this study was approved by the Institutional Review Board of the Ohio State University Wexner Medical Center under Study Number: 2004C0124 and the Ohio State University's OHRP Federal wide Assurance #00006378. No human subjects were directly consented for this study as the tissues were obtained from a biorepository.

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