Exploring the role of obesity-induced extracellular vesicles secretion and associated oncogenic proteins in endometrial cancer pathogenesis.

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Abstract

Endometrial cancer (EC) is the leading gynecologic malignancy in the United States with obesity implicated in 57% of cases. This research delves into the molecular complexities of extracellular vesicle (EV) secretion as carriers of oncogenic protein expression and their involvement in obesity-mediated EC. An understanding of these mechanisms is pivotal for unraveling pathways relevant to obesity-associated EC, thereby guiding the development of innovative prevention and treatment strategies. Our investigation revealed a significant increase in EV secretion carrying oncogenic proteins (TMEM205, STAT5, and FAS) in adipose and uterine tissues/serum samples from obese EC patients compared to their non-obese counterparts. We identified alterations in EV-regulating proteins (Rab7, Rab11, and Rab27a) in obesity-mediated EC patient adipose and uterine samples. Through a 24-week analysis of the effects of a 45% kcal high-fat diet (HFD) on mice, we observed heightened body weight, increased adipose tissue, enlarged uterine horns, and heightened inflammation in the HFD group. This correlated with elevated levels of EV secretion and increased expression of oncogenic proteins TMEM205, FAS, and STAT5, while the tumor suppressor gene PIAS3 was downregulated in adipose and uterine tissues in HFD treated mice. Furthermore, our study confirmed that adipocyte derived EVs increased EC cell proliferation and migration. Additionally, we identified that the small molecule inhibitors (HO-3867) or Metformin inhibited EV secretion in vitro and in vivo, demonstrating significant inhibition of high glucose or adipocyte-mediated EC cell proliferation and a reduction in body weight and adipose tissue accumulation when administered to HFD mice. Moreover, HO-3867 or Metformin treatment inhibits HFD induced hyperplasia by altered the expression of EV-regulated proteins (Rab7, Rab11, and Rab27a) and decreased oncogenic protein expression (TMEM205, FAS and STAT5) levels. This study provides critical insights into the mechanisms supporting obesity-mediated EV secretion with oncogenic protein expression, shedding light on their role in EC pathogenesis. Additionally, it offers pre-clinical evidence supporting the initiation of novel studies for EV-targeted therapies aimed at preventing obesity-mediated EC.

Introduction

Endometrial cancer is the most common gynecologic malignancy in developed countries and ranks as the sixth most common cancer among women globally[1, 2]. The recent rise in endometrial cancer cases is linked to the growing obesity epidemic in the developing world. A recent meta-analysis demonstrated a significant increase in endometrial cancer risk with each 5kg/m2 increase in BMI (RR 1.59, 95% CI 1.59–1.68), particularly for type 1 endometrial cancers. Women with class III obesity with a BMI ≥ 40 kg/m2 face an increased lifetime risk of development of developing cancer to 10–15% when compared to 2% in the overall general population[3]. Obesity also influences oncologic outcomes with doubling the risk of cancer-specific mortality[4]. These associated morbidities lead to an important area for understanding obesity-related biomarkers role in development of cancer and development of possible screening. The mechanisms behind the increased risk involve a complex milieu including insulin resistance, chronic inflammation, elevated sex steroid hormones, and adipokines.
Obesity raises the risk of endometrial cancer in both pre- and post-menopausal individuals through multiple mechanisms. Increase in amount of adipose tissue causes increase in circulating estrogen levels by conversion of androgens by aromatase thereby increasing endometrial cell growth and gene transcription\[5\]. This effect is more pronounced when progesterone is lacking either in an ovulatory state or in post-menopausal states. Chronic inflammation is another consequence of excess visceral fat and results in various issues like high insulin levels, elevated blood sugar, and reduced anti-inflammatory cytokines. Some proposed mechanisms involve changes in how fat tissue functions, causing alterations in the secretion of adipokines and extracellular vesicles (EVs) [6–8]. Recent studies indicate that obese individuals’ fat cells release EVs containing proteins that may raise cancer risk [9–11]. EVs are tiny vesicles (30–120 nm) released by various cells and those from adipose tissue playing a crucial role in communication between adipocytes and nearby cells\[12–14\]. However, it not clearly elucidated how obesity-related signaling pathways involving EV secretions might affect the development of endometrial hyperplasia and subsequent progression to carcinoma. Recently, our group identified TMEM205, a novel transmembrane protein, and Rab11 expression as expressed proteins in obese associated EC tissues. Further, TMEM205 is overexpressed in EC samples in the TCGA dataset\[15\]. In addition, basic and clinical studies have linked high levels of TMEM205 targeting protein of Rab11 expression with EVs secretion, cancer progression, and poor patient survival\[16, 17\]. Elucidating the molecular mechanism of obesity mediated oncogenic protein expression which regulates the EVs secretion through Rab11 in in the context of obesity associated EC is essential for developing alternative strategies for prevention and therapeutic targeting for EC including drug resistant tumors.

We have developed a new type of compound with a diarylidenyl-piperidone (DAP - HO-3867) core structure combined with an N-hydroxypyrroline (-NOH) component which can turn into a nitroxide. These compounds are effective at inhibiting STAT3, TMEM205 and FAS, an important factor in our research [18–20]. Notably, DAP compounds have a unique pattern of toxicity – they target cancer cells but leave healthy cells unharmed. This is due to the -NOH group, which protects healthy tissues from harm. At the same time, the compound remains toxic to cancer cells, making it a promising therapy. DAP-HO-3867 and Metformin has shown great effectiveness in fighting endometrial cancer in a high-fat diet-induced model and in primary endometrial cancer cells. These results suggest that blocking EVs secretion pathway proteins (TMEM205 and STAT3) by HO-3867 or Metformin could be a safe and targeted treatment for obesity-related endometrial cancer.

**Materials & Methods**

**Cell lines and cultures**

The Ishikawa (grade 1) and HEC-1 endometrial cancer cell line was used in this study (Obtained from ATCC). The cells were grown in DMEM with either low (1 mM), normal (5 mM), or high (25 mM) concentrations of glucose. The media was supplemented with 10% heat-inactivated FBS, 2% sodium pyruvate, 1% penicillin, and 1% streptomycin. Cells were grown in a 75 mm flask to 70% confluence at 37
°C in an environment of 5% CO₂ and 95% air. Cells were trypsinized (0.05% trypsin/EDTA) routinely. We confirmed mycoplasma activity using ATCC® Universal Mycoplasma Detection Kit in all cell lines every 2 months. Once the frozen cells were thawed, they were passaged for a maximum of 5 times and discarded thereafter and a fresh vial was thawed.

**High fat Diet (HFD) study in immunocompetent mice:** C57BL/6 Female immunocompetent mice were procured from the Charles River, MA, USA. The mice were categorized into two groups: the Normal Chow Diet group (NCD group, n = 10) and the High Fat Diet group (HFD group, n = 12). These mice were age-matched and cohabitated in well-ventilated cages with solid flooring, grouped in 2–3 individuals. They were provided unrestricted access to food and water on a 24-hour basis. The NCD group adhered to a normal chow diet throughout the study until reaching twelve months of age. The selected diet for the NCD group contained 3% kcal fat content, recognized as the standard diet for research mice. The HFD group, on the other hand, was immediately transitioned to a high fat diet upon arrival at three months of age, maintaining this diet until the conclusion of the study at twelve months of age. The high fat diet comprised 45% kcal fat (Research Diets, NJ, USA). At 25 weeks of age, magnetic resonance imaging (MRI) was conducted, followed by euthanasia and systematic harvesting of animal organs. In the therapeutic study, female mice were stratified into five groups (NCD, HFD, HFD + HO-3867, HFD + Metformin, and HFD + HO + Metformin) for distinct treatments. The treatments commenced after 16 weeks of high fat diet exposure, spanning 8 weeks, after which the mice were sacrificed. Uterine, ovary, and adipose tissues were collected for subsequent molecular analysis.

**Transmission Electron microscopy (TEM):** The uterine and adipose tissues were processed for TEM imaging as follows: The tissues were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for at least 24 hours at 4°C. Likewise. The adipose and uterine tissues were washed and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes at room temperature. Both tissue samples were postfixed with 1% osmium tetroxide and then enbloc stained with 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Eponate 12 epoxy resin (Ted Pella Inc., Redding, CA). Ultrathin sections were cut with a Leica EM UC6 ultramicrotome (Leica microsystems Inc., Deerfield, IL), collected on copper grids. Images were acquired with an FEI Technai G2 Spirit transmission electron microscope (Thermo Fisher Scientific, Waltham, MA), and a Macrofire (Optronics, Inc., Chelmsford, MA) digital camera and AMT image capture software.

**RNA isolation and Reverse Transcription PCR (RT-PCR)**

Total RNA was isolated from tissues or EC cells samples using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA samples with an optical density A260/A280 ratio between 1.8 and 2.1 were used. RT-PCR was then performed using the Transcriptor First Strand Complementary DNA (cDNA) Synthesis Kit (Roche Diagnostics, Indianapolis, IN, USA) to synthesize cDNA. RT-PCR was performed with 1mg of RNA template. The reaction was carried out using the Veriti Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific) and random hexamer primers.
Isolation of EVs using SEC columns

IZON qEV original size exclusion columns (Izon Science) were used in the isolation EVs. The columns were first removed from 4°C and the 20% ethanol storage solution was allowed to run through the column followed by 20 ml particle-free PBS. Serum samples were diluted to 500 µl with sterile filtered particle-free PBS and the sample was overlaid on the qEV size exclusion column followed by elution with particle-free PBS. The flowthrough was collected in 500 µl fractions, and fractions 4–7 were pooled for further downstream analysis such as Image stream flowcytometry. For ELISA plating the pooled EV samples were further concentrated using an Amicon Ultra-0.5 ml (10 kDa) centrifugal filter device (Merck Millipore) for protein estimation and relative quantification of potential EV biomarker proteins across different patient samples.

Statistical analysis: Results were expressed as mean ± S.D. Comparisons between groups were made by a Student’s t-test. The significance level was set at p ≤ 0.05.

Results

Identifying the extracellular vesicles secretion and regulated proteins in obesity associated EC patient samples.

In previous studies, EVs have been recognized as critical players in cancer progression and metastasis. Our research focused on characterizing EVs, quantifying their levels, and identifying key proteins involved in their secretion pathways in endometrial cancer, particularly in patients with obesity-related cancer. Using advanced Image Stream Flow Cytometry Analysis (ISA), our findings demonstrated a significant 3- to 5-fold increase in EVs release in the serum of obese EC patients compared to non-obese EC patients (Fig. 1A & B, Sup. Figure 1). Additionally, we examined protein expression profiles related to EV secretion pathways and noted a substantial upregulation of proteins like Rab7, Rab11, and Rab27a in obese endometrial cancer patients’ tissues samples compared to their non-obese counterparts (Fig. 1C). Furthermore, our investigation uncovered distinct protein expression patterns in obesity-related EC which includes elevated levels of oncogenic proteins such as TMEM205, STAT5, as well as FAS. Similarly, there was an identified reduction in the tumor suppressor protein PIAS3 (Fig. 1D). These findings emphasize the pivotal role of increased EVs levels in obesity-related endometrial cancer and highlight specific protein expression changes in affected patients. Furthermore, they present potential opportunities for early biomarkers and therapeutic approaches for individuals dealing with endometrial cancer related to obesity.

Determining the supplementation of high fat diet (HFD) induced endometrial hyperplasia through the EVs secretion and alteration of target proteins.

To investigate the impact of continuous High-Fat Diet (HFD) consumption (45 kcal% fat diet) on the secretion of Extracellular Vesicles (EVs) and the regulation of oncogenic proteins in adipose and uterine tissues, we initiated our study by assessing body weight and uterine morphology in mice following 24 weeks of HFD treatment. As expected, mice on the HFD exhibited significantly higher body weight and
increased adipose tissue deposition compared to the control group (Fig. 2A-C, Sup. Figure 2). Notably, the HFD group displayed pronounced enlargement of uterine horn size (hyperplasia) (Fig. 2D & E), accompanied by observable inflammation and heightened cell proliferation in the endometrial layer (Fig. 2F).

Subsequently, we explored whether the HFD had an impact on the EVs secretion phenotype in adipose and uterine tissues. At the 24-week mark, we sacrificed mice from the HFD group and conducted Transmission Electron Microscopy (TEM) analysis. Our observations revealed an increase in EVs secretion and the formation of multi-vesicular bodies in adipose and uterine tissues from the HFD-treated mice (Fig. 3A, Sup. Figures 3 & 4), significant elevation of EVs secretion was observed in HFD mice serum samples (Fig. 3B & C). Furthermore, there was an upregulation of key EVs regulation genes, including Rab11 and Rab27a in HFD treated adipose and uterine tissues (Fig. 3D & E).

Additionally, our investigation unveiled that HFD supplementation was associated with altered EVs secretion patterns and the expression of oncogenic proteins (specifically TMEM205, FAS, and pSTAT5). Notably, we observed a downregulation of the tumor suppressor gene PIAS3 in adipose and uterine tissues (Fig. 4A & B, Sup. Figures 5 & 6). The cumulative evidence from our study indicates that the changed expression of oncogenic proteins under high-fat diet (HFD) conditions contributes to cellular proliferation, hyperplasia, tumor initiation, and the aggressive phenotype typical of endometrial cancer. Consequently, our data strongly suggest that the obese tissue microenvironment induced by HFD plays a pivotal role in both the initiation and progression of EC through a notable rise in extracellular vesicle release among obese patients with endometrial cancer. This increase is accompanied by distinction protein expression patterns linked to oncogenic transformation and the progression of cancer.

**Identifying the adipocytes and high glucose treated increase EVs secretion and its role in endometrial cancer cells proliferation and migration.** Scientific evidence demonstrating the regulatory role of adipocytes and adipocyte-mediated signaling pathways in cancer initiation and progression, including obesity-associated EC progression. Our research reveals that adipocytes and their mediated signaling pathways play a pivotal role in the regulation of various cancer initiation and progression events, particularly in the context of obesity-associated EC progression. Notably, our study observed that the differentiation of adipocytes (Fig. 5A & B, Sup. Figure 7) leads to the secretion of EVs carrying oncogenic proteins. Co-culturing adipocyte secreted EVs (Fig. 5C) with EC Ishikawa (IK) cells resulted in a significant increase in EC cell proliferation (Fig. 5D & E). Additionally, our recent investigations have highlighted the impact of high glucose levels on EC cell proliferation, causing alterations in oncogenic proteins and microRNAs [11]. In this study, we sought to elucidate the effects of high glucose-mediated EV secretion on EC cell proliferation and migration by investigating the up-regulation of specific oncogenic proteins, namely TMEM205, FAS, and STAT5. Ishikawa endometrial cancer cells were cultured in low, normal, or high glucose media for 24–72 hours, observations indicated that high glucose-treated EC cells exhibited enhanced proliferation and migration compared to cells treated with regular glucose (Fig. 5F & G).

Importantl, when we blocked EV secretion using established EV inhibitors or small molecule inhibitors in EC cells, we observed a significant reduction in EC cell proliferation (Fig. 5H-J). These findings strongly
suggest a direct link between obesity-mediated EV secretion and its contribution to the pathogenesis of EC. The results also highlight the potential therapeutic significance of targeting EV secretion to inhibit EC cell proliferation, offering novel insights into the development of strategies for combating this disease.

The impact of EVs secretion inhibitor compound HO-3867 and Metformin on HFD induced endometrial hyperplasia for prevention of EC.

To explore the impact of inhibiting HFD-mediated EVs secretion and the modulation of oncogenic proteins in adipose and uterine tissues, our study employed a small molecule inhibitor targeting EVs regulatory pathway proteins (STAT3, STAT5, and TMEM205) \[21–23\], along with Metformin to inhibit EV secretion, the administration of HO-3867 and Metformin showed a notable trend towards reduced body weights and adipose tissue accumulations. However, the differences observed in the combined treatment of the high-fat diet (HFD) mice group did not reach statistical significance. (Fig. 6A & B, Sup. Figure 8). Importantly, the total body weight of the mice at the conclusion of the experiment was similar between the treatment and control (untreated) groups (Fig. 6C), indicating that animals treated with HO-3867 and Metformin did not exhibit any apparent signs of toxicity based on the body weight profile. Furthermore, electron paramagnetic resonance (EPR) spectra obtained from adipose and uterine tissue biopsies of HFD-treated mice revealed the presence of HO-3867 in its oxidized (nitroxide) form (Fig. 6D). Adipose and uterine tissues collected were subjected to ELISA or reverse transcription-polymerase chain reaction (RT-PCR), demonstrating a decrease in the protein expression of TMEM205, FAS, STAT5, c-MYC, Cyclin D2, VEGFR, and an increase in tumor suppressor protein in Metformin and HO-3867 treated versus control mice (Fig. 6E, Sup. Figures 9 & 10). Additionally, we observed an increase in EVs secretion levels and an upregulation of key EVs regulatory genes, including Rab7 and Rab11, and a decrease in Rab27a in HO-3867 and Metformin treated adipose and uterine tissues (Fig. 6F & G). These results indicate that both HO and Metformin effectively decrease the expression of oncogenic proteins and suppress the secretion of exosomes within the context of HFD-induced endometrial hyperplasia and the simulation of endometrial cancer.

Discussion

In our current research, we have achieved several significant new findings: (i) Obesity-associated endometrial cancer (EC) patients and mice exhibited markedly elevated levels of extracellular vesicle (EV) secretion containing oncogenic proteins in their serum and tissues samples; (ii) We observed alterations of oncogenic proteins (TMEM205, STAT5 and FAS), as well as the tumor suppressor protein PIAS3 in samples of adipose and uterine tissues from EC patients and high-fat diet (HFD) mediated obesity mice; (iii) Adipocytes or high glucose mediated EVs secretion play a key role in EC cells proliferation/migration; (iv) Our DAP derivative compound HO-3867 and Metformin effectively inhibits blocks EV secretion in both in vitro and in vivo mouse models, resulted in the inhibition of the growth of obesity-associated EC, both in vitro and in vivo.
Although the link between obesity and the onset of endometrial cancer, progressing to EC, is widely acknowledged, the specific cellular mechanisms by which obesity influences this progression remains unclear. Various potential pathophysiological mechanisms have been suggested, encompassing alterations in the physiological function of adipose tissue, resulting in chronic inflammation, and changes in the secretion patterns of adipokines and extracellular vesicles [24–26]. Recent investigations have unveiled the role of adipocytes from obese patients in secreting EVs carrying oncogenic proteins, thereby increasing the incidence of cancer [9–11]. EVs, originating from adipose tissue, play a pivotal role in mediating communication between adipocytes and neighboring cells [27–29]. Nevertheless, the impact of obesity-mediated signaling pathways involving EVs secretion on the development of EC and its progression to carcinoma remains a subject of uncertainty. Our study presents compelling evidence that highlights the critical role of obesity-associated EVs carrying oncogenic proteins, including TMEM205, STAT5, and FAS in the pathogenesis of EC. These EV-borne oncogenic proteins have the potential to fuse with adjacent cells, instigating a cascade of events that ultimately result in the downregulation of tumor suppressor proteins within both adipose and uterine tissues. This downregulation sets the stage for increased cellular proliferation, thus initiating the growth of EC. Previous reports show that in various cancers, and including obese cancer patients, increased EVs secretion directly corresponds to elevated expression of oncogenic proteins [30–32]. Our current findings provide valuable insights into how EV-mediated signaling plays a pivotal role in the complex interplay between obesity and the development and progression of EC.

While the oncogenic role of STAT5, FAS and TMEM205 in cancer progression is established, its involvement in obesity-associated EC remains unexplored. Importantly, this protein has been shown to govern the regulation of extracellular vesicle (EV) secretion under conditions of obesity. A recent study has revealed that STAT3 or TMEM205 co-localizes with EV secretion proteins, specifically Rab8 or Rab11 [21, 33, 34], and its expression is upregulated in ovarian cancer cells, where it plays a crucial role in regulating EV secretion. Drawing from our knowledge of the roles of GTPases such as Rab7, Rab11, and Rab27 in vesicle trafficking [35–37]. The upregulation of oncogenic proteins (STAT5, TMEM205 and FAS) expression may lead to its co-localization with Rab7, Rab11, or Rab27, subsequently enhancing the rate of vesicle recycling following increased EV secretion in the context of obesity-associated adipose and endometrial tissues. This process ultimately results in elevated EV secretion containing oncogenic proteins, contributing to the growth of endometrial cancer in obese patients. Nevertheless, further studies are warranted to investigate into the mechanisms behind TMEM205, STAT5 and FAS upregulation and its interaction with Rab family proteins, as well as its role in regulating Rab11 or Rab27a and increased EV secretion in obesity-associated endometrial cancer.

Previous studies have established that the inhibition of extracellular vesicle (EV) secretion using diuretic agents like Amiloride (AME) or small molecule inhibitors such as GW4869 can effectively reduce the growth of pancreatic, lung, and colon cancers [38–42]. However, the potential impact of inhibiting proteins involved in EV secretion pathways or blocking exosome secretion as a means of prevention or oncotherapy in the context of obesity-mediated endometrial cancer (EC) has not been previously explored. We have developed a small molecule inhibitor, diarylidenylpiperidones-NOH (DAP-HO-3867). HO-
3867 demonstrates selective inhibition of EVs secretion in cancer cells while sparing normal cells. The HO-3867 molecule incorporates an N-hydroxypyrroline (NOH), which serves as a nitroxide precursor, functioning as a cytotoxicity modulator[18]. This unique feature imparts antioxidant protection to noncancerous tissues, making HO-3867 a potentially safe inhibitor for EVs regulating oncogenic proteins (STAT3, FAS and TMEM205), with applications in preventing obesity-mediated EC and other cancers within an obese microenvironment.

While a consensus regarding the definitive cancer prevention or anticancer effects of HO-3867 in endometrial cancer, both in vitro and in vivo, remains elusive, the findings from our current study suggest a dual-pathway theory that focuses on targeting EVs secretion pathway proteins in obesity-associated EC. Our in vivo investigations employing diverse animal models hold the potential to substantiate whether the inhibitory impact of HO-3867 or Metformin on the EV secretion pathway significantly contributes to their overall cancer prevention. These forthcoming studies may also unveil novel therapeutic applications for inhibiting EVs secretion in the ongoing battle against cancer.

Declarations

**CONFLICT OF INTEREST:** The authors declare no conflict of interest.

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**Author Contributions**

K.S. designed all experiments. T.S., and R.Z, performed all the in vivostudy experiments, RT-PCR, ELISA and IHC assays analyzed and the data collected. K.D.PD analyzed EVs and adipocytes experiments. W.K and J. W assisted in collecting the human samples and EVs or target proteins analyzed in human samples. D.K. performed anti-BRDU assay in HGM treated cells. M.A and A.S performed all the in vivo tumor, histopathology, and imaging work. L.M. provided the additional EC samples. C.C, D.O, D.E.C and H.K, provided the valuable suggestion and support in human subject study. T.S, W.K, and K.S. wrote, edited, and proofread the manuscript.

**Disclosure of Potential Conflicts of Interest:** No potential conflicts of interest were disclosed.

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
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**Figures**

Figure 1

**A**

Control | Obese EC | Non-Obese-EC
--- | --- | ---
Exosomes | Exosomes | Exosomes

**B**

Bar graph showing EVs concentrations (cells/ml).

**C**

Relative gene expression of Rab7a, Rab11a, and Rab27a.

**D**

Relative gene expression of TMEM205, STAT5, FAS, and PIA3S.
Enhanced extracellular vesicle (EV) secretion associated with oncogenic proteins and EV regulatory proteins in endometrial cancer (EC) patient samples.

(A & B) Serum samples from obese EC and endometrial benign patients were thawed on ice and diluted (1:4 to 1:10) in 1xPBS. EVs were isolated from 50µL of patient serum using the Exoquick kit. EV concentration was assessed by Image Stream Flow Cytometry (ISF), revealing a significant increase in EV concentration and fold change in obese EC patients compared to non-obese EC (n=5, p<0.01).

(C) Regulatory proteins of EVs, including Rab7, Rab11, and Rab27a, were analyzed by RT-PCR in non-obese EC early-stage and obese EC early and late-stage tissue samples (n=5, p<0.001 or 0.005).

(D) Targeting oncogenic proteins (TMEM205, STAT5, FAS, and PIAS3) and their relative gene expression levels were examined in EC patient samples by RT-PCR (n=5, p<0.001 or 0.005).
Figure 2

High-Fat Diet (HFD) supplementation promotes adipose tissue accumulation and induces endometrial hyperplasia.

(A & B) Immunocompetent mice were treated with a high-fat diet (HFD, 45% protein calorie) for 24 weeks. Changes in body weight were assessed at the beginning (8 weeks) and end (24 weeks) of the HFD
treatment (n = 10/group, p<0.05). Representative images depict accumulated levels of adipose tissue (circled) and morphological changes in the uterus, which is enlarged in mice fed a HFD for 24 weeks.

(C & D) Increased accumulation of adipose tissue and uterine enlargement were observed in mice fed a HFD compared to controls (n=5, p<0.005).

(E) Enhanced cell proliferation in the endometrium of mice fed a HFD compared to controls (normal diet) is illustrated. The inset shows a higher magnification of the increased cell proliferation.
Figure 3

Increased extracellular vesicle (EV) secretion associated with EVs regulatory proteins in HFD treated mice samples.

(A) Transmission Electron Microscopy (TEM) reveals increased formation of EVs in uterine tissues of high-fat diet (HFD) treated and control diet mice.
(B & C) Quantification of EVs in serum samples from control and HFD-treated obese mice using ImageStream analysis.

(D & E) Analysis of relative protein and gene expression levels in adipose and uterine tissues from HFD and control diet-treated mice. Protein expression was determined by ELISA, while gene expression levels were assessed by RT-PCR.
Figure 4

Alterations in oncogenic and tumor suppressor proteins in High-Fat Diet (HFD) treated mice tissues.

(A & B) Analysis of targeted proteins (TMEM205, STAT5, FAS, and PIAS3) and their relative gene expression levels in adipose and uterine tissues of HFD and control diet-treated mice. The assessment was conducted using a combination of RT-PCR, ELISA, and Immunohistochemistry (IHC) techniques (n=5, p<0.01 or 0.005).
Figure 5

Adipocytes and high glucose mediated EVs secretion increased EC cells proliferation and migration. (A) Adipose-derived mesenchymal stem cells underwent adipocyte differentiation initiated 72 hours post-confluency on Day 3, 5, and 7, followed by maintenance in adipocyte medium until day 15. Microscopic images (10x and 40x) at different time points show increasing lipid accumulation.
(B) To confirm adipocyte formation, cells were cultured in a 12-well plate, fixed, and stained with 0.2% oil red O in 2-propanol for 10 minutes at room temperature. Adipogenesis was measured at various time points.

(C) Adipocyte-mediated extracellular vesicle (EV) secretion and size confirmation by ISF.

(D & E) Cell proliferation assessed by 5-ethynyl 2’-deoxyuridine (EdU) incorporation using flow cytometry in IK cells co-cultured with adipocyte-released EVs, demonstrating an increased percentage of EdU+ cells compared to control cells (untreated EVs) (n=4, p < 0.05 or 0.01).

(F & G) IK or HEC13 cells treated with regular or high glucose (HGM) medium for 24 to 72 hours; cell proliferation measured by anti-BrdU assay and migration assessed by wound healing assay (n=4, p < 0.01 or 0.005).

(H & I) EV secretion levels analyzed and quantified by ISF in EV inhibitors Amiloride (10uM), GW4869 (5uM), Metformin (10uM), or HO-3867 (5uM) for 24 hours in EC cells.

(J) Cell proliferation assay measured in IK cells treated with small molecule inhibitors HO-3867 or Metformin for 24h (n=5, p < 0.01).
Figure 6

Effects of Metformin and HO-3867 on EVs secretion in HFD-Fed Mice: prevention of endothelial cancer.

(A & B) Representative images depicting the accumulated levels of adipose and uterine tissues in mice fed a high-fat diet (HFD) for 24 weeks, relative to those fed a control diet or treated with HO-3867 or Metformin (5 mg/kg in drinking water or food).
(C) Reduction in body weight observed in HFD-fed mice following treatment with HO-3867 or Metformin.

(D) Electron paramagnetic resonance (EPR) spectra obtained from adipose and uterine tissue biopsies of HFD-treated mice, illustrating the presence of HO-3867 in its oxidized (nitroxide) form. The levels of HO-3867 in the tissue samples were quantified using EPR (n=4; **: p<0.005).

(E & F) Relative expression levels of target genes determined by RT-PCR in Metformin- or exosome inhibitor (HO-3867)-treated uterine tissues of HFD-fed mice (n=5, p<0.05).

(G) Isolation of EVs from HFD and DAP-5 treated mice serum samples, with quantification of EVs secretion levels by interfacial-sensitive fluorescence (ISF) (n=4; p<0.001).

**Supplementary Files**

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- ObesitySup.FigureFinal.pdf