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School of medicine
Department of Health Sciences

Master's degree course in MEDICAL BIOTECHNOLOGIES

Graduation Thesis

Title

In vitro investigation of the role of adipose-derived mesenchymal stem cells (AD-MSCs) in supporting the progression of endometrial cancer

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Contents

1. ABSTRACT

Rationale of the study: Endometrial cancer (EC) stands as the most prevalent gynecologic malignancy in developed countries and ranks as the sixth most common cancer among women globally. Distinguished by two primary types based on histological and molecular characteristics, type I EC is predominantly estrogen-dependent with a generally favourable prognosis, while type II EC, characterized by non-estrogen-dependent pathways and genetic alterations, exhibits a more aggressive nature and poorer outcomes. Among the risk factors obesity, is linked to an increased risk of EC through mechanisms involving hormonal imbalances, insulin resistance, and chronic inflammation, all contributing to the disease's pathogenesis. The relationship between adipose tissue, particularly visceral fat, and EC involves complex interactions within the tumour microenvironment (TME), however, the role of adipose-derived mesenchymal stem cells (AD-MSCs) which, under the influence of obesity-induced inflammation, may support tumour growth and progression remains poorly understood.

Planning of the Study: This study aimed to understand the biological dynamics between visceral AD-MSCs and EC cell lines. Using a co-culture methodology, we investigated how AD-MSCs modulate key aspects of EC behaviour, including proliferation, migration, and response to chemotherapy. Additionally, we explored the metabolic alterations occurring within the TME by investigating mitochondrial activity and lipid metabolism in EC cells co-cultured with AD-MSCs.

Results: Our comprehensive analysis revealed that AD-MSCs significantly amplify the proliferative and migratory capabilities of EC cells. Notably, co-cultured EC cells demonstrated a marked increase in resistance to paclitaxel chemotherapy, suggesting a profound AD-MSC-mediated mechanism underlying chemoresistance. Further examination of mitochondrial function showed a significant upregulation in mitochondrial membrane potential in EC cells co-cultured with AD-MSCs, particularly pronounced in the HEC1A cell line, indicating enhanced mitochondrial activity and possibly increased energy production. Lipid metabolism studies revealed enhanced intracellular lipid levels within both AD-MSCs and EC cells in the co-culture, suggesting a bidirectional metabolic reprogramming that promotes a lipid-rich TME advantageous to cancer progression.

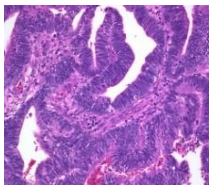
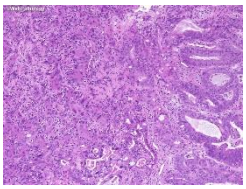
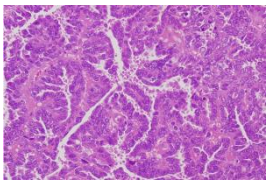
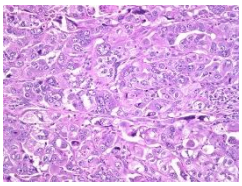
Conclusion: The findings support that visceral AD-MSCs are likely to drive EC progression. These observations focus on the need for further exploration to develop innovative strategies for EC screening, diagnosis, and treatment, particularly in the context of obesity-related malignancies. Targeting the interplay between AD-MSCs and EC cells or the molecular mediators secreted by AD-MSCs presents a promising approach for enhancing the efficacy of cancer therapeutics and managing EC and related hormone-driven cancers.

2. INTRODUCTION

2.1 Endometrial cancer (EC)

Endometrial cancer (EC) is the sixth most common cancer in women worldwide and the most common gynecologic malignancy in the developed world [1]. EC is characterized by the abnormal growth of glandular elements of the endometrium and is classified as type I or type II based on its histological characteristics (**Table 1**) [2]. Type I ECs are estrogen-driven and have endometroid differentiation, while type II ECs are not estrogen-dependent and are classified as non-endometrioid (serous, clear cell, mucinous) [2]. Type I ECs represent approximately 70-80% of all ECs [1] and tend to have a more favorable prognosis than type II cancers, which are usually more aggressive and consequently associated with poorer prognosis [3]. These distinct subtypes arise from distinct molecular pathways, type I EC are predominantly driven by unopposed estrogen exposure, and type II ECs are driven by genetic alterations, including mutations in TP53, p53, and DNA polymerase epsilon [3]. Understanding the underlying molecular and pathological characteristics of these subtypes is crucial for accurate diagnosis, prognosis, and personalized therapeutic strategies.

Table 1*Clinicopathological and molecular characteristics of the common types of epithelial endometrial carcinoma (adapted from Murali R, and Soslow R, 2014*)

	 **A**	 **B**	 **C**	 **D**
Histological type	Endometrioid	Endometrioid	Serous	Clear cell
Histological grade	Low	High	High	High
Metastasis	Uncommon	Lymph nodes Distant organs	Lymph nodes Peritoneal Distant organs	Lymph nodes Peritoneal –/+
Prognosis	Favorable	Poor	Poor	Poor
Molecular markers				
ER/PR expression	+	+/–	–/+	–
PTEN expression	–/+	–/+	+	+
DNA MMR loss	–/+	–/+	–	–/+
Aberrant P53	–	–/+	+	–/+
Ki-67/MIB-1	Low	High	High	Low or high

2.1.1 Epidemiology

EC is the sixth most common cancer in women worldwide, with a prevalence of 25.1 cases per 100,000 individuals [4]. The highest incidence is observed in North America and Eastern Europe (**Figure 1**), where approximately 55,000 and 100,000 new cases are diagnosed annually, respectively EC generally has a favorable prognosis, with a 5-year survival rate exceeding 90% for localized disease [5]. However, survival rates decline significantly for women with regional (68%) or distant (17%) spread. EC primarily affects postmenopausal women, with a median age of 63 at diagnosis. Less than 10% of cases occur in women under 50 [6]. Geographical and racial variations in incidence have been observed. In England, for instance, black women have the highest incidence, while South Asian women have the lowest [7]. Interestingly, in the United States, African American women have a lower incidence compared to white women [8].

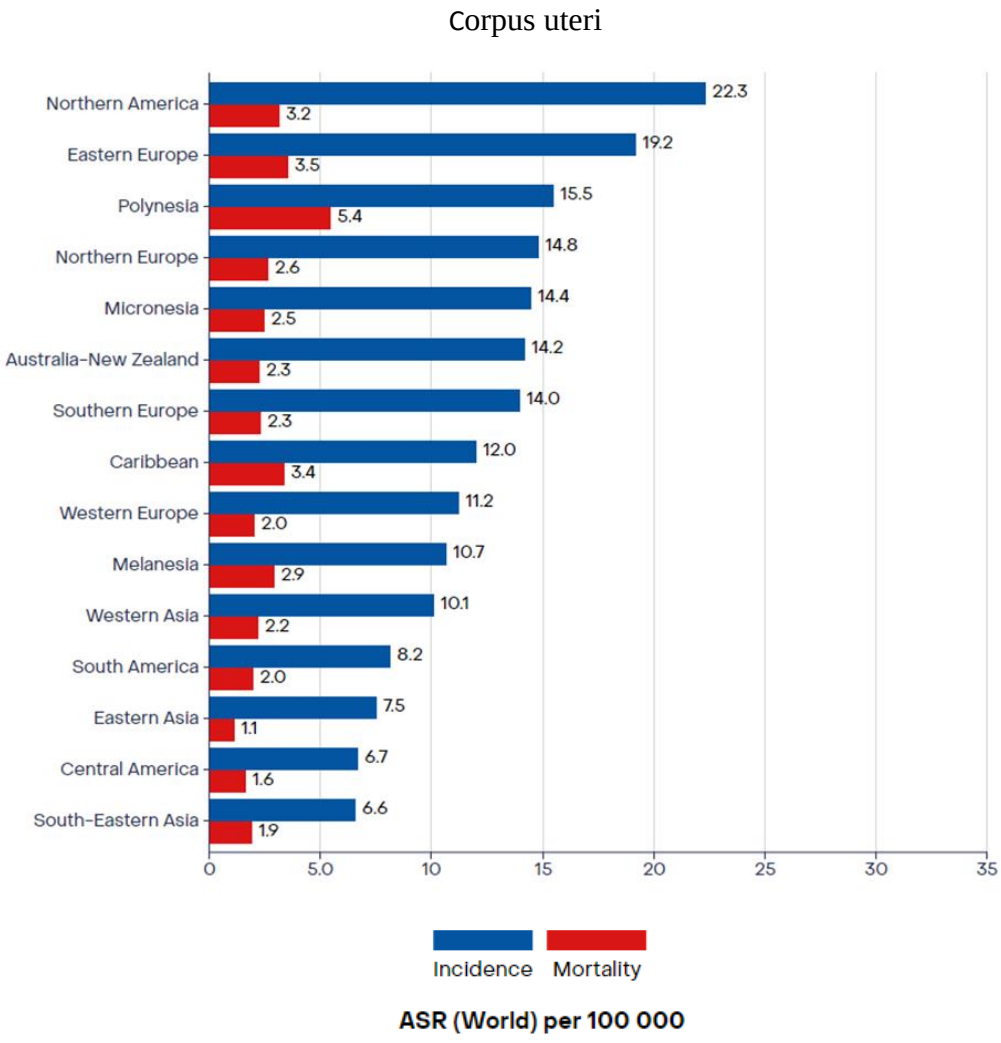


Figure 1 *Age-Standardized Rate (World) per 100 000 incidence and mortality rates for uterine corpus cancer by regions in 2022; adapted from Global Cancer Observatory.*

Most EC are sporadic, but Lynch syndrome is the most common familial form [9]. Women with Lynch syndrome have an increased risk of both EC and colorectal cancer (30-70% and 25-70%, respectively) [9]. They tend to develop EC at a younger age (median age of 46-62 years) compared to women in the general population [7]. Endometrial carcinoma is typically classified into two types: type I (endometrioid) and type II [10]. Type I is the most prevalent subtype (80-90%) and is associated with a favourable prognosis (**Table 2**). It is estrogen-dependent, grows slowly, and metastasizes late [10]. Type II, instead, is estrogen-independent, grows more rapidly, and metastasizes earlier, leading to a poorer prognosis [10]. Serous and clear-cell carcinomas belong to type II, as well as approximately 25% of high-grade endometrioid carcinomas [10], [11]. The proportion of type II cancer is higher in women with Lynch syndrome compared to women in the general population but type I remains the predominant type [12]. Worldwide incidence rates of EC have been increasing, particularly in the twenty-first century, where age-standardized incidence rates have increased from 6.5 per 100,000 in 2002 [13] to 8.2 per 100,000 in 2012 [4]. Furthermore, Type I ECs have been increasing in the U.S. and Europe [13] This increased incidence of EC can likely be attributed to changes in lifestyle risk factors (e.g., diet, sedentary behavior, and use of hormone replacement therapy), which are all strongly associated with EC risk [14].

Table 2 *Properties of type I and type II EC (Zhou Q, 2018)*

	Type I	*Type II*
Median age at presentation	63	67
5-year survival rate	85%	58%
Histology	Endometrioid Grade 1, 2, 3 (75%)	Papillary serous Clear cell Carcinosarcoma Undifferentiated carcinoma Endometrioid G3 (25%)
Subtype frequency	80-90%	10%
Estrogen-dependent	Yes	No
Genetic alterations	PTEN, KRAS, CTNNB1, PI3CA, MSI an MLHI	TP53 (mainly serous)
Known risk factors	Metabolic syndrome, obesity, Type II DM, unopposed estrogen	No known risk factors

2.1.2 Risk factors

EC is the second most common malignancy in women, after breast cancer, in developed countries. In 2012, global statistics recorded 319,605 new cases and 76,160 deaths attributed to EC [15]. From 1978 to 2013, there has been a consistent and gradual increase in age-standardized incidence and mortality rates of EC in most developed nations[15]. Multiple risk factors for EC have been identified (Table 3). The risk of EC rises with age, with a predominant diagnosis occurring postmenopause [16]. Older women diagnosed with EC often present with higher-grade and more advanced-stage tumors compared to their younger counterparts [17]. Additional risk factors for EC include prolonged exposure to unopposed estrogens, elevated postmenopausal estrogen concentration, a history of breast cancer, and a first-degree family history of EC [18]. Among EC patients, factors contributing to higher mortality include pre-diagnosis obesity, type 2 diabetes mellitus, and heart disease [19], [20]. The World Cancer Research Fund Continuous Update Project panel has established convincing evidence linking body weight to an increased risk of EC [21]. Obesity stands out as one of the most potent risk factors for developing EC [22]. It is strongly hypothesized that the rising global prevalence of obesity, especially in developed countries, is a significant contributor to the overall increase in EC incidence [23], [24]]. Diagnosis often occurs post-menopause with the peak incidence observed around the seventh decade of life [25]. A minority, approximately 5% of women, will develop adenocarcinoma before reaching 40 years of age, and a notable 20-25% receive the diagnosis before experiencing menopause [25].

Table 3 *Risk factors of EC (adapted from Eleanor R Jones, 2021)*

Risk factors	Preventive factors
Increasing age	High level of physical activity
Obesity	Healthy diet
Hypertension	Oral contraceptive use
Diabetes 2	Weight loss
Unopposed estrogen	Late menarche
Nulliparity	Parity
Late menopause	Hormone replacement therapy

2.2 The link between obesity and cancer

Obesity is a global, complex, multi-factorial, and generally preventable disease [26]. The global incidence of obesity has doubled in the last 40 years; nowadays more than one-third of the world population is classified as obese or overweight [27]. If this trend persists, experts predict that by the year 2030, the estimates will exceed 50%[28]. The World Health Organization (WHO) has defined obesity as “abnormal or excessive fat accumulation to the extent that health may be impaired” [29] and it results from a complex of genetical, environmental, socioeconomic, and behavioral factors [26]. The body mass index (BMI) is utilized to measure excess body fat and obesity is commonly defined as a BMI or weight-to-height ratio equal to or exceeding 30kg/m2 [29]. Recently, there has been more evidence suggesting that abdominal fat rather than total body fat is an independent risk factor for cancer-related and cardiovascular outcomes. The presence of visceral fat, which surrounds organs and is metabolically active, is believed to contribute to metabolic dysregulation, predisposing individuals to various diseases. The involvement of adipose tissue and its surrounding environment in the process of carcinogenesis, metastasis development, and disease progression was previously reported [30]. However, the complicated mechanisms driving carcinogenesis remain complex and not fully understood disrupted fatty acid secretion and metabolism, alterations in the extracellular matrix, the release of anabolic and sex hormones, immune system dysregulation, persistent inflammation, and modifications in the gut microbiome are implicated in the initiation and progression of cancer in the context of obesity (Figure 2) [31].

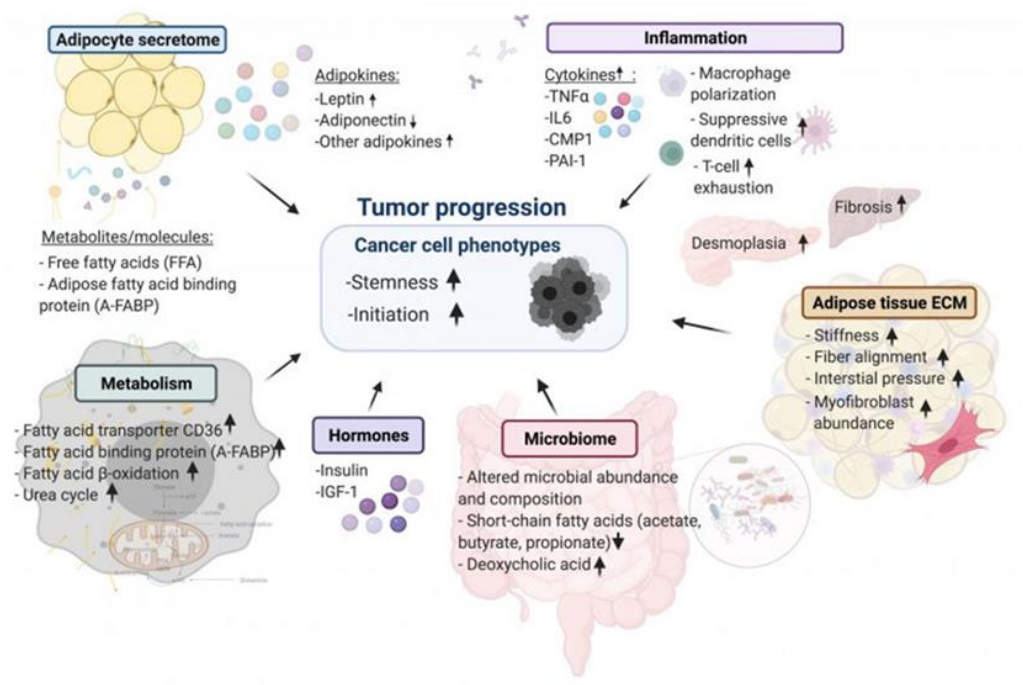


Figure 2 *Summary of cellular mechanisms linking cancers to obesity. (Adapted from Xiao-Zheng Liu, 2021)*

Obesity is linked to an increased susceptibility to cancer, mainly due to three underlying biological mechanisms [31]. The first of these mechanisms is related to the concept of adipose tissue acting as an “organ”, with the capacity to release chemical mediators and enzymes [32]. The overproduction of estrogen by adipose tissue has been associated with an increased risk of developing breast, endometrial, ovarian, and other cancers [33]. The second mechanism involves hyperinsulinemia resulting from increased BMI. This condition stimulates insulin’s normal growth function and extends the duration of action of insulin-like growth factor-1 (IGF-1) [34]. Higher levels of insulin and IGF-1, common in obesity, contribute to insulin resistance, a well-known cancer risk factor. The association between hyperinsulinemia and the development of colon, renal prostate, and ECs has been established [35]. The third mechanism focused on the pro-inflammatory environment created by the altered secretion of adipokines by adipose tissue. Increased levels of inflammatory adipokines, such as leptin, known for its inflammatory, proliferative, and anti-apoptotic properties, contribute to chronic inflammation in adipose tissue [36]. On the contrary, low levels of adiponectin, another adipokine with antiproliferative properties, are observed in obese individuals with a healthy weight [36]. This chronic inflammation triggers carcinogenesis and cancer progression and is supported by pre-clinical studies [36]Furthermore, obesity is associated with altered levels of inflammatory cytokines and increased prevalence of inflammatory diseases, contributing to oxidative stress, DNA damage, and a higher likelihood of developing biliary tract, liver, and other malignancies [37]. Moreover, obesity may enhance cancer risk by impairing tumour immunity, altering the mechanical characteristics of surrounding tissues, and influencing factors like adipokines, immune cell modulation, systemic inflammation, angiogenesis, metabolic changes, extracellular matrix modulation, and extracellular vesicles such as exosomes, all implicated in metastasis [38].

2.3. Obesity and EC

Obesity significantly correlates with the onset of EC, with an estimated 57% of cases in the United States linked to being overweight or obese [39]. The risk of EC rises with increasing body mass index (BMI), showing a 50% escalation for every 5-unit increase in BMI[40]. While endometrioid EC is most prevalent and strongly associated with obesity (Figure 3), other aggressive types (serous, clear cell, and carcinosarcoma) also exhibit an increased incidence with higher BMI [27], [41]. Additionally, obesity is linked to adverse outcomes for women with EC, with higher mortality rates, particularly for morbidly obese individuals [40]. Despite the established evidence connecting EC and obesity, public awareness of this association remains limited. Recent research highlights the

role of visceral fat, a complex endocrine organ composed of various cells (adipocytes and preadipocytes, infiltrating macrophages, stromal cells, nerve cells, and stem cells.), in promoting endometrial proliferation and tumorigenesis [41]. Visceral fat secretes adipokines, influencing both localized and systemic effects, and serves as a source of mesenchymal stem cells that support tumour growth[42], [43]. In premenopausal women, ovarian-driven cyclic estrogen expression stimulates endometrial proliferation [44].

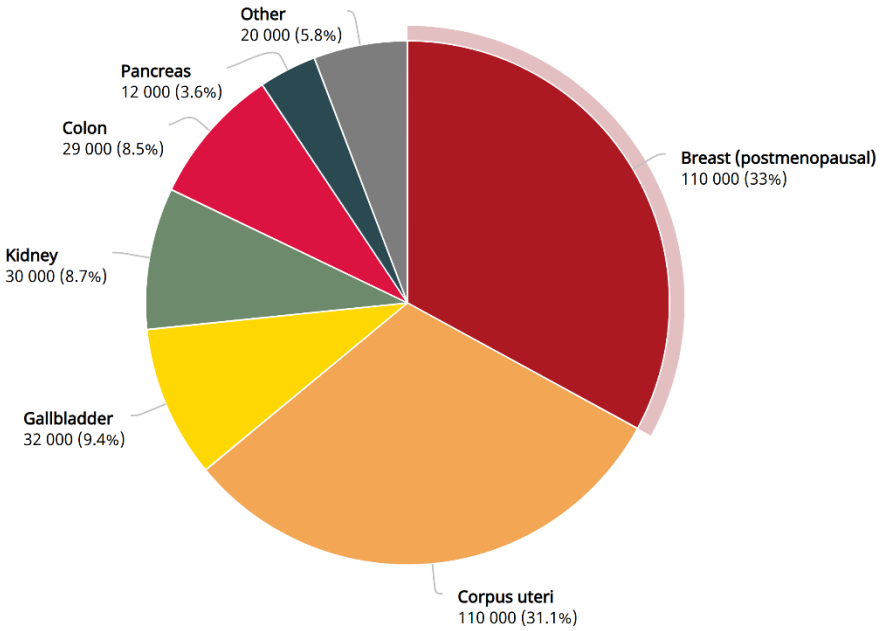


Figure 3 *Cancer cases (at all anatomical sites) among females (worldwide) in 2012 attributable to excess body mass index, shown by anatomical site as percentages of the total number of all such attributable cases at all anatomical sites in this population.*

Postmenopause, adipose tissue becomes the primary site of estrogen synthesis, with aromatase produced by adipocytes, preadipocytes, and mesenchymal stem cells contributing to estrogen-induced endometrial proliferation [45]. Increasing adiposity decreases sex hormone-binding globulin (SHBG), elevating bioactive estrogen levels. Estrogen, bound to receptors, directly modulates the transcription of pro-proliferative genes, including IGF1R and IGF1[46]. G-protein–coupled estrogen receptor 1 (GPER1) mediates non-genomic signalling, activating the MAPK and AKT pathways to stimulate endometrial proliferation [45], [46]. Estrogen, functioning both as a mitogen and a mutagen, generates genotoxic metabolites that react with DNA, potentially leading to genetic instability [47]. ECs often exhibit DNA mismatch repair defects, suggesting a role for

estrogen metabolites in tumorigenesis [47]. Hyperactivity of insulin and IGF1 signalling, prevalent in type 2 diabetes, contributes to EC pathogenesis [48]. Increased receptor expression heightens responsiveness to insulin and IGF1, promoting MAPK and PI3K/AKT/mTOR signalling [49]. Loss of the PTEN tumour suppressor gene amplifies proliferative signalling, and hyperglycemia fuels the growth of metabolically active tissue, including endometrial hyperplasia and cancer [50]. Adipose tissue, rich in adipokines, plays a crucial role in metabolism and inflammation. Proinflammatory adipokines associated with obesity, such as leptin and interleukin 6, contribute to insulin resistance and endometrial proliferation [50]. Adipokines influence SHBG expression, affecting systemic estrogen levels. Adiponectin, an anti-inflammatory adipokine, induces SHBG synthesis, while proinflammatory cytokines, like tumour necrosis factor α, are linked to low plasma SHBG levels, increasing EC risk [51], [52]. Adipokine-mediated inflammation leads to cellular stress, genetic instability, and DNA damage, particularly in conditions of DNA mismatch repair defects, fostering endometrial hyperplasia and cancer [52]. This comprehensive understanding emphasizes the intricate interplay between obesity, hormonal factors, and genetic mechanisms in the development of EC.

2.4. Adipose tissue, mesenchymal stem cells, and EC relationship

Obesity induces modification in the composition, structure, and functionality of adipose tissue, leading to inflammation and metabolic dysfunction [53] (Figure **4**). Adipose tissue is rich in mesenchymal stem cells or adipose stem cells (MSCs/ASCs), and the effects of obesity alter the characteristics and activities of these cells. AD-MSCs are a type of multipotent stem cell that can differentiate into various cell types, including adipocytes, osteoblasts, and chondrocytes [54]. Found in various tissues such as bone marrow, umbilical cord blood, and adipose tissue, AD-MSCs are known for their self-renewal abilities, higher proliferative capacity, and the ability to secrete bioactive molecules that can influence different mechanisms [55]. Normally, AD-MSCs exhibit anti-inflammatory actions via inhibition of the proliferation of dendritic cells, natural killer cells, T and B lymphocytes [56]. Additionally, AD-MSCs affect innate and specific immune cells, and research has shown an ability to suppress tumour growth [57]. Due to their ability to differentiate into multiple lineages and exhibit diverse beneficial effects such as anti-inflammatory, immunoregulatory, anti-apoptotic, and anti-fibrotic properties, AD-MSCs have been studied for treating various diseases, including cancer [58]. However, the therapeutic application of AD-MSCs is complicated because of their heterogeneous nature [54]. For instance, the potency of AD-MSCs is influenced by the microenvironment in which they reside [59]. Metabolic syndromes like diabetes

and obesity, which alter systemic and local environments, significantly affect the behaviour and function of AD-MSCs. This impairment in obese-derived AD-MSCs is believed to be partly attributed to the chronic inflammatory environment, as pro-inflammatory cytokines exert detrimental effects on MSC proliferation, activation, and regenerative abilities, [59]. Additionally, cellular processes occurring in the adjacent adipose tissues, such as metabolic reprogramming, oxidative stress, and hypoxia, contribute to early senescence, apoptosis, and a compromised immunomodulatory function of AD-MSCs [53]. However, the primary determinant influencing the fate of AD-MSCs during obesity condition remains unclear.

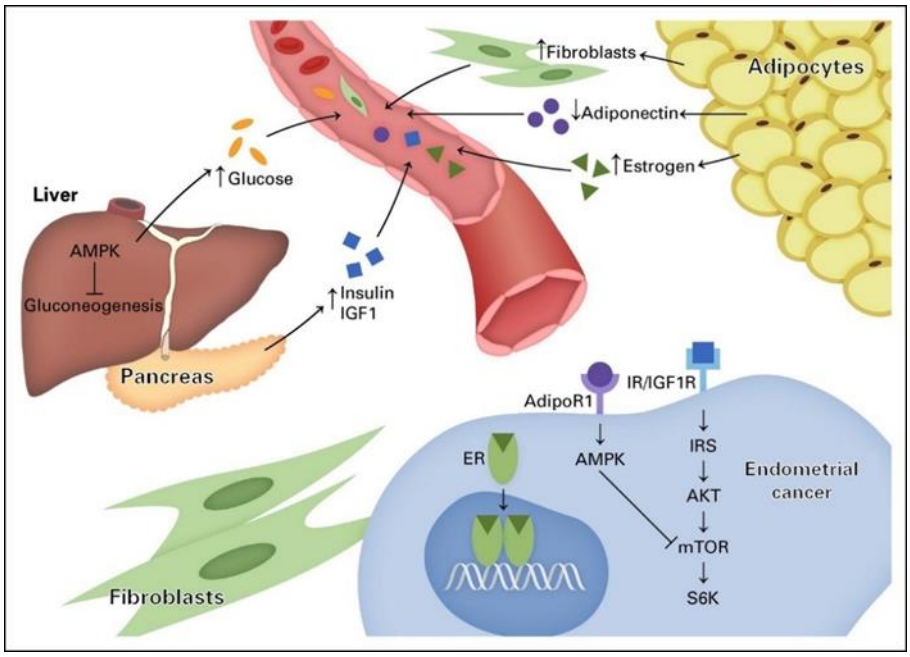


Figure 4 *Illustration of the impact of obesity on EC proliferation and tumorigenesis. Obesity influences the increased risk of endometrial cancer through various mechanisms. The increased adiposity triggers greater aromatase activity, converting androgens to estrogens, directly promoting endometrial proliferation, and activating proliferative gene transcription. Visceral adiposity-associated chronic inflammation, mediated by proinflammatory adipokines, results in hyperinsulinemia, elevated insulin-like growth factor 1(IGF1), and hyperglycemia, all contributing to increased endometrial proliferation. Simultaneously, there is a decrease in anti-inflammatory cytokines. This inflammation and elevated metabolites contribute to DNA damage and genetic instability. Additionally, stem cells from adipose tissue are recruited, participating in the formation of a supportive tumor microenvironment. A key element in this process includes estrogen receptor (ER), insulin receptor substrate (IRS), and mammalian target of rapamycin (mTOR). (adapted from Michaela A. Onstad, 2016)*

2.4.1 Lipid metabolism and EC

Lipid metabolism plays a crucial role in various physiological processes, including cell membrane formation, energy storage, and signalling pathways [60]. Dysregulation of lipid metabolism has been implicated in the development and progression of various cancers including EC [61]. Dysregulated fat metabolism stands out as a prominent metabolic alteration in tumour biology, fundamental for the acquisition of energy, signalling molecules, and other biological processes contributing to the microstructural basis required for cell proliferation, survival, invasion, metastasis, and the overall influence on tumour behavior and response to cancer treatments [61]. Metabolic reprogramming is recognized as a growing hallmark of cancer. cancer cells often produce adenosine triphosphate (ATP) through aerobic glycolysis, known as the Warburg effect [62]. Conversely, the “reverse Warburg effect” occurs when cancer cells utilize energy generated by stromal cells in the tumour microenvironment [61]. Apart from glucose, cancer cells also uptake free fatty acids and glycerol from stromal adipocytes [62]. Additionally, tumour cells depend on stromal sources for metabolic substrates like lactate, glutamine, and fatty acids, activating glycolysis and lipolysis pathways in stromal cells [63]. While the role of glucose metabolites in cancer progression is well established, the contribution of lipid metabolites is less clearly defined. Reprogramming of lipid metabolism is a crucial aspect of the energy metabolism alterations observed in cancer cells. Adipocytes, by storing triglycerides through lipogenesis and producing diacylglycerol, monoacylglycerol, and free fatty acids via lipolysis, play a role in regulating energy balance throughout the organism [64], [65]. Highly proliferative cancer cells satisfy their energy needs by either producing lipids and cholesterol internally through lipogenesis or acquiring them from the tumour microenvironment (TME) by inducing lipolysis in adipocytes [65]. To explore the metabolic interaction between adipocytes and tumours, different in vitro co-culture studies involving cancer cells and adipocyte, or adipocyte-conditioned medium have been done [62]. Scientists have reported that co-culture induces lipolysis in adipocytes through hormone-sensitive lipase (HLS) and adipose triglyceride lipase (ATGL), leading to the release of free fatty acids transferred to neighbouring cancer cells as an energy source [62]. Additionally, a reduction in lipid droplet size and number has been observed in cancer-associated adipocytes (CAA) [66]. These free fatty acids can serve as substrates for mitochondrial β-oxidation or support cancer cell proliferation and migration [67]. Elevated levels of lipid metabolites have been reported in various cancers, including breast, prostate, glioblastoma, endometrial and hepatocellular carcinoma [67]. The Fatty Acid Binding Protein (FABP) family proteins, actively involved in lipid metabolism, show increased expression during cancer progression [68]. Specifically, FABP4, facilitating fatty acid transport, is elevated in breast cancer [68]. Furthermore, cancer cells utilize lipids for cell

membrane synthesis, generation of lipid-derived bioactive molecules, and production of exosomes [69],[52]. Free fatty acids and glycerol released from lipolysis contribute to the biosynthesis of membrane lipids during cancer proliferation [69]. Bioactive lipids, such as steroid hormones, diacylglycerol, and phospholipids, play a role in the reprogramming of cancer cells [70]. A study conducted on breast cancer reported that the fatty acid receptor CD36 is implicated in the initiation of metastasis in breast-derived tumours and is associated with poor prognosis [70]. Adipocyte-derived exosomes, referred to as adiposomes, have been shown to stimulate cell invasion and migration in certain cancers such as melanoma [52].

2.4.2 The impact of obesity-induced chronic inflammation on AD-MSCs in the tumor microenvironment.

Chronic inflammation, a critical consequence of obesity, plays a crucial role in the development and progression of EC. In the obesity state, chronic inflammation has a profound impact on the behavior of AD-MSCs [73] within the tumor microenvironment, and EC represents a critical instance of this interaction. Obesity becomes a source of sustained inflammatory signals due to the secretion of adipokines and cytokines such as leptin, TNF-α, and IL-6 [74]. These molecules generate a state of chronic inflammation that can pervade the systemic circulation and local tissue environments, including the endometrial lining where they can drastically influence the tumorigenic process [55]. In EC, the chronic inflammatory state induced by obesity may reprogram AD-MSCs, which are inherently present in abundance within adipose tissue, to support tumour progression [75]. These cells, typically involved in tissue homeostasis and repair, may instead adopt pro-tumorigenic roles under the influence of persistent inflammatory stimuli [76]. They can become key contributors to the formation of a tumor-supportive stroma by enhancing angiogenesis, modulating immune responses, and promoting tissue remodelling, which collectively facilitates tumour growth and spread (**Figure 5**) [77]. The altered AD-MSCs within the obese tumour microenvironment of EC can secrete a myriad of factors that promote tumour cell survival and proliferation [42]. This secretion profile includes increased levels of vascular endothelial growth factor (VEGF), which drives angiogenesis, and transforming growth factor-beta (TGF-β), which can suppress local immune responses against tumour cells [77]. Moreover, the chronic inflammatory environment can encourage the migration of AD-MSCs to the tumour site, where they can interact with and possibly convert into tumor-associated fibroblasts (TAFs), further enhancing the cancer-supportive environment [55]. The recruitment of these cells to the tumour site is facilitated by chemokines released within the inflammatory environment, and once present within the tumour, AD-MSCs may

aggravate the inflammatory state [75]. The feedback loop created by this interaction can lead to the generation of a progressively hospitable niche for tumour cells, with the AD-MSCs contributing to a microenvironment that is permissive and even supportive of tumour growth and metastasis [75]. Moreover, the obesity-associated inflammatory environment can stimulate AD-MSCs to produce extracellular matrix-modifying enzymes, facilitating cancer cell invasion and spreading [75]. In EC, this can be particularly detrimental, as the ease of cancerous cell migration can lead to higher stages of disease at diagnosis and poorer prognosis [73]. By understanding and potentially modulating the inflammatory influences on AD-MSCs, strategies can be developed to mitigate their tumor-supportive actions, offering new opportunities for the treatment of obesity-associated EC [73], [75] .

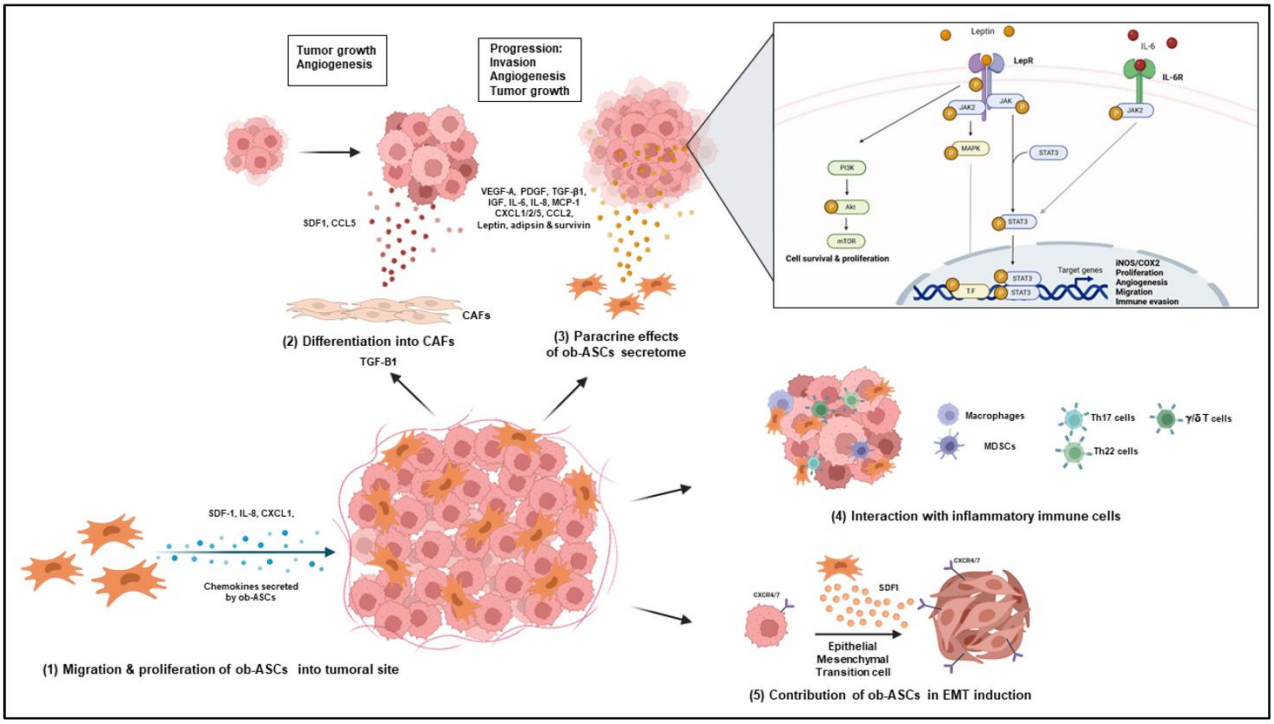


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Figure 5 *AD-MSCs play a critical role in advancing cancer progression. These cells have the capacity to cross to tumor locations, transform into carcinoma-associated fibroblasts (CAFs), and release factors that promote tumor growth. Such interactions can lead to enhanced tumor development, the formation of new blood vessels (neo-angiogenesis), increased invasiveness, and the potential for epithelial-mesenchymal transition (EMT), all of which contribute to the progression of cancer (Adapted from the work of Pestel Julien,, 2023).*

3. OBJECTIVE OF THE THESIS

Obesity is recognized as an important risk factor for cancer development, especially for those driven by hormones such as EC. Adipocytes have been recognized for their role in cancer development, but the involvement of AD-MSCs in the process of carcinogenesis, especially concerning EC, remains less clear. The central aim of this project is to understand the interaction between EC cell lines, both estrogen-dependent (i.e., Ishikawa) and estrogen-independent (i.e., HEC1A) and AD-MSCs, with a specific interest in the in vitro characterization of the influence of AD-MSCs on EC cells biology and metabolism.

4. MATERIALS AND METHODS

4.1 AD-MSCs isolation

AD-MSCs were isolated from human visceral adipose tissue biopsy samples collected from patients treated at the Division of Surgery of Ospedale Maggiore della Carità, Novara. The study protocol was approved by the Novara Ethical Committee (CE 10-21, 14.01.2012), and written informed consent was obtained from all participants. Biopsy samples were initially washed with phosphate-buffered saline (PBS) containing antibiotics and antimycotics (penicillin, streptomycin, amphotericin, Sigma Aldrich). Subsequently, the samples were mechanically fragmented in 5mL of Dulbecco's Modified Eagle Medium (DMEM) and enzymatically digested at 37°C for 1 hour in 10 mL of DMEM supplemented with collagenase (1 mg/mL). Following digestion, the samples were filtered through a sterile cell strainer and centrifuged for 5 minutes at 300g. The resulting stromal vascular fraction (SVF) was then cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Euroclone) and 1% antibiotics and antimycotics for further expansion and experimentation.

4.2 Cell culture

AD-MSCs (MSC3 and MSC4) and EC (Ishikawa and HEC1A) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) and 1% antibiotics/antimycotics (Penicillin, Streptomycin, Amphotericin, Sigma). Cells were maintained at 37°C in a 5% CO2 humidified atmosphere, with medium changes performed every 3 days and subculturing conducted prior to reaching confluency.

4.3 MTT (Thiazolyl Blue Tetrazolium Bromide) Viability Assay and chemoresistance

EC cells were plated at a density of 2,500 cells per well in a 96-well plate. For the long-term chemoresistance assay, EC cell suspensions containing 1000 cells in 50 μL of DMEM supplemented with 10% FBS were seeded into a 96-well plate. Paclitaxel (Taxol, Onxal, Cayman Chemical) solution at a concentration of 0.17 μM was added to the treatment groups and incubated for 20 hours. The vehicle alone served as the control. Thiazolyl blue tetrazolium bromide (MTT) was added at various time points to assess cell viability, following the procedure described earlier.

Thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, Saint Louis, MO, USA) at a concentration of 0.5 mg/mL was added to each well and incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, the formazan crystals were dissolved using 100 μL of acidic isopropanol (2 N HCl), and the absorbance was measured at 570 nm and 650 nm using a spectrophotometer

(Victor, PerkinElmer, Waltham, MA, USA). Data analysis and visualization were performed using GraphPad Prism 9.0 software.

4.4 ADSCs and EC cells co-cultures

The wells of a 12-well plate were divided into two halves using soft agar solution (25 mg/mL), with 200 µL deposited in each well using a pipette and allowed to dry for 1 hour before plating. Adipose-derived stem cells (ADCs) were seeded onto one-half of the divided well at a density of 5000 cells/100 µL, while endothelial cells (EC cells) were seeded onto the remaining half of the same plate at a density of 2500 cells/100 µL. The plates were then carefully transferred to the incubator until cell attachment occurred. Afterwards, the medium was aspirated, and cells were washed with 500 µL of PBS, followed by the addition of 800 µL of DMEM supplemented with 10% FBS. Cells were cultured for 2 weeks in the incubator at 37°C with 5% CO2, with medium changes performed every three days. For control experiments, the same numbers of ADSCs and EC cells were seeded on each side of the divided well.

4.5 Migration assay

Cell migration assays were conducted using Culture-Inserts (IBIdi). In brief, 25,000 cells in 50 µL of EC cell suspension were plated on each side of the insert in a 24-well plate and incubated at 37°C with 5% CO2. Phase-contrast images were captured immediately after removing the insert and at 24 hours, 48 hours, and 72 hours thereafter. Subsequently, wound areas were quantified using ImageJ software (NIH, Bethesda, MD), and data analysis was performed using GraphPad Prism 9.0.

4.6 Colony formation assay

EC cells (100 cells/well) were seeded in 24-well plates containing 500 µL of DMEM supplemented with 10% FBS and cultured for 8 days. For crystal violet staining, the plates were placed on ice, and the medium was aspirated and washed with ice-cold PBS 1X. Subsequently, 300 µL of methanol was added to each well, and the plates were kept at -20°C for 15 minutes. Afterwards, the plates were again washed with ice-cold PBS, and 300 µL of 0.2% crystal violet solution was added to each well and incubated for 15 minutes at room temperature. Following incubation, the plates were washed with ice-cold ultrapure water until the crystal violet staining disappeared. Images were captured using a phone camera and analyzed using ImageJ software (NIH, Bethesda, MD). Data analysis was performed using GraphPad Prism 9.0.

4.7 Nile red staining

For lipid analysis, cells participating in the coculture, and control cells were stained with a mix containing Nile Red (1mg/mL) and DAPI (1 µg/mL). Nile Red, a fluorescent dye, is utilized for detecting and measuring cellular lipids, while DAPI serves as a stain for DNA, emitting blue fluorescence when it binds to DNA sequences. The procedure began with the cultivation of EC cells and AD-MSC, followed by to discard of the growth medium. Subsequently, cells are fixed using 300 µL of 4% paraformaldehyde (PFA) for 20 minutes at ambient temperature in a dark environment. After fixation, the cells were washed with 1X PBS and then incubated with the staining solution in darkness at room temperature for half an hour. Fluorescence microscopy (using the Floid Cell Imaging Station from Life Technology) was employed to capture the signals, and ImageJ software was utilized for image analysis.

4.8 JC1 Dye for mitochondrial membrane potential

To assess mitochondrial membrane potential, cells were labelled with JC1 fluorescent dye, which forms red J-aggregates on healthy mitochondrial membranes. These aggregates emit fluorescence with a shift from green (approximately 529 nm) to red (approximately 590 nm). Thus, a decrease in the red/green intensity ratio indicates mitochondrial depolarization due to reduced red J-aggregate formation. The JC1 solution was prepared in DMEM without FBS at a ratio of 1:100. AD-MSCs and EC cells were seeded at a density of 2500 cells/100 µL in a 12-well plate as previously described. After two weeks of coculture, cells were stained with JC1 dye for 30 minutes in the dark at 37°C. Subsequently, cells were washed with PBS, and 300 µL of DMEM supplemented with 10% FBS was added. Fluorescent signals were captured using a fluorescence microscope (FLoid Cell Imaging Station, Life Technologies), and images were analyzed using ImageJ software. The red/green fluorescence ratio was calculated using GraphPad Prism 9.0.

5. RESULTS

5.1 AD-MSCs promote chemotherapy resistance and proliferative potential in cocultured EC cells

Recent studies highlight the important role of the tumour microenvironment, especially AD-MSCs, which is crucial in cancer dynamics, influencing tumour growth, metastasis, and response to therapy [71]. Thus, the investigation of AD-MSCs is likely to be crucial to understanding EC progression in obese patients. Within this framework, we cocultured EC cells with AD-MSCs for 14 days and we monitored cell proliferation and drug resistance over a time frame of 7 days as displayed in **Figure 7A**. Our experimental plan included, two different cell lines, Ishikawa (**Figure 7B**) and HEC1A (**Figure 7C**), and four experimental conditions: cells cultured alone (***Alone***), cells co-cultured with mesenchymal stem cells (*CoC*), cells treated with the chemotherapeutic agent paclitaxel (***Alone+Paclitaxel***), and cells co-cultured with AD-MSCs and treated with paclitaxel (*Coc+Paclitaxel*).

Both EC cell lines cocultured with AD-MSCs demonstrated an increased proliferative potential compared to control cells except HEC1A cocultured with MSC3 indicating possible cell line-specific interactions with the AD-MSCs.

Notably, in all the tested cell lines treatment with 0.17 μM paclitaxel displayed significant cytotoxicity only in control cells while cocultured cells maintained their proliferative potential demonstrated by a rebound in proliferation, particularly evident at day 4 and 7. Suggesting that coculture with AD-MSCs supports chemoresistance in EC cells with HEC1A cells maintaining a balanced response across all conditions, with less variation compared to the Ishikawa cell line.

Altogether our data suggest that the proximity of EC cells and AD-MSCs supports EC cells proliferation and induces chemoresistance.

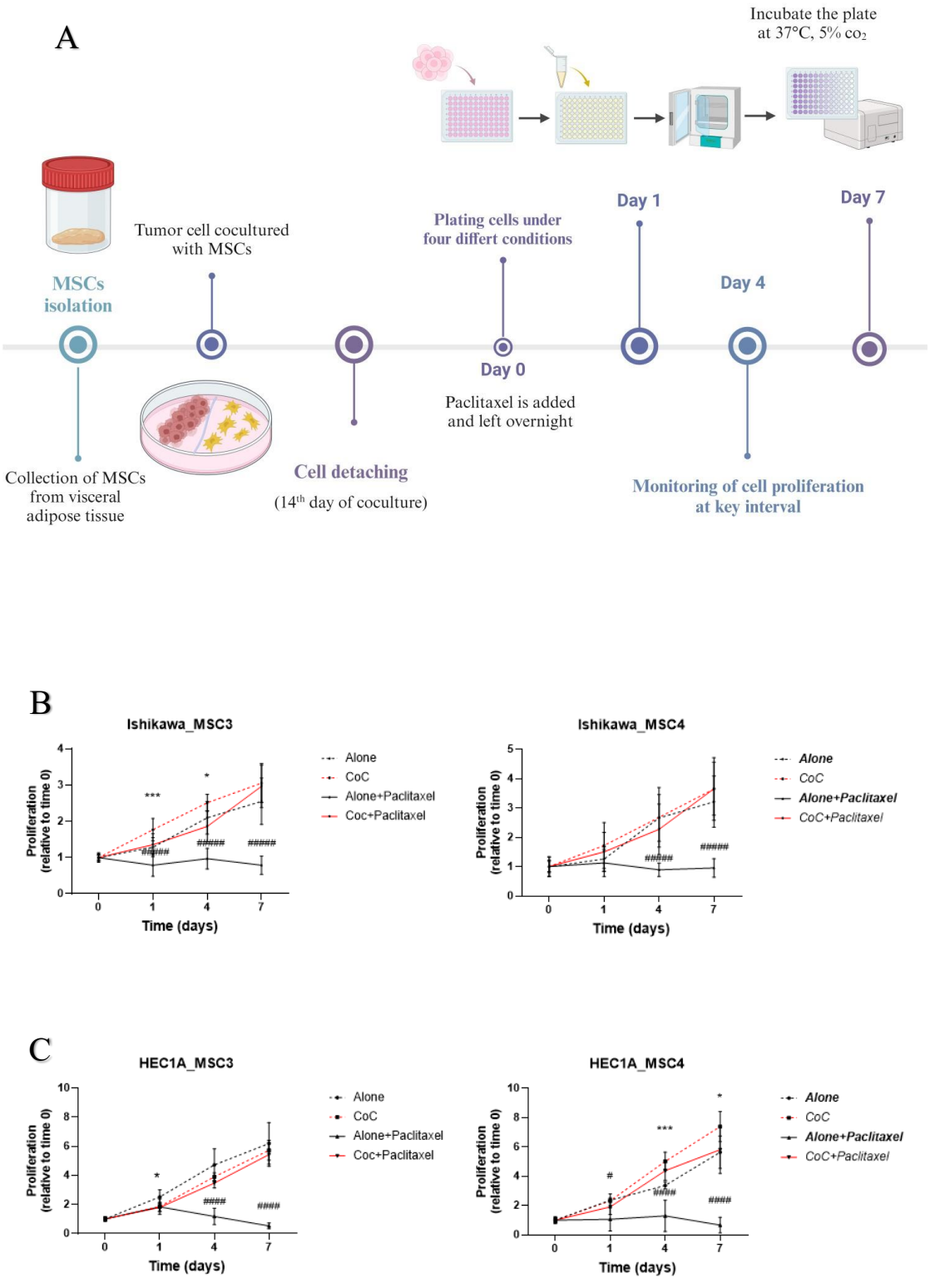

Figure 6 EC cells co-culture with AD-MSCs exhibit enhanced proliferation and chemoresistance. *Experimental overview of proliferation and chemoresistance (**A**). Time course assessment of cocultured EC cells proliferation and sensitivity to paclitaxel: Ishikawa cells (**B**) and HEC1A cells (**C**). Proliferation was normalized to time 0. Data represent the mean ± SD of at least 3 independent experiments performed in triplicate. Student's t-test: * p<0.05, *** p<0.001 cocultured cells vs control cells (Alone) dotted lines, and ### p<0.001, #### p<0.0001 co-cultured cells with paclitaxel (Coc+Paclitaxel) vs paclitaxel-treated control cells (Alone+Paclitaxel) solid lines.*

5.2 Cocultured EC cells displayed increased migration potential

Our results suggest that EC cells develop a more aggressive behaviour when interacting with AD-AD-MSCs. To explore the altered characteristics of co-cultured EC cells, we assessed their ability to migrate by conducting wound healing assays over a period time frame ranging from 0 to 72 hours. Migration was evaluated by analysing the wound area percentage after 0, 24, 48, and 72 hours to monitor the closure rate, a well-known indicator of cell migration [79]. In both Ishikawa and HEC1A cell lines (**Figure 7 A, B**), when cultured alone, the wound area decreased over time as the cells migrated to close the space. This closure rate was slower compared to when the cells were previously co-cultured with AD-MSCs. The coculture with MSC3 and MSC4 consistently appeared to enhance the migration of both cell lines, as indicated by a more significant reduction in the wound area, particularly noticeable at 48 and 72-hour (**Figure 7 C**). In conclusion, data suggest that co-culturing Ishikawa or HEC1A cells with MSCs promotes cell migration, an important factor in the process of wound healing. This could be indicative of the enhanced invasive potential of these cancer cell lines when influenced by the tumour microenvironment, potentially mediated by interactions with AD-MSCs. This observation is crucial for understanding the mechanisms of cancer progression and the impact of the cellular microenvironment on therapeutic outcomes.

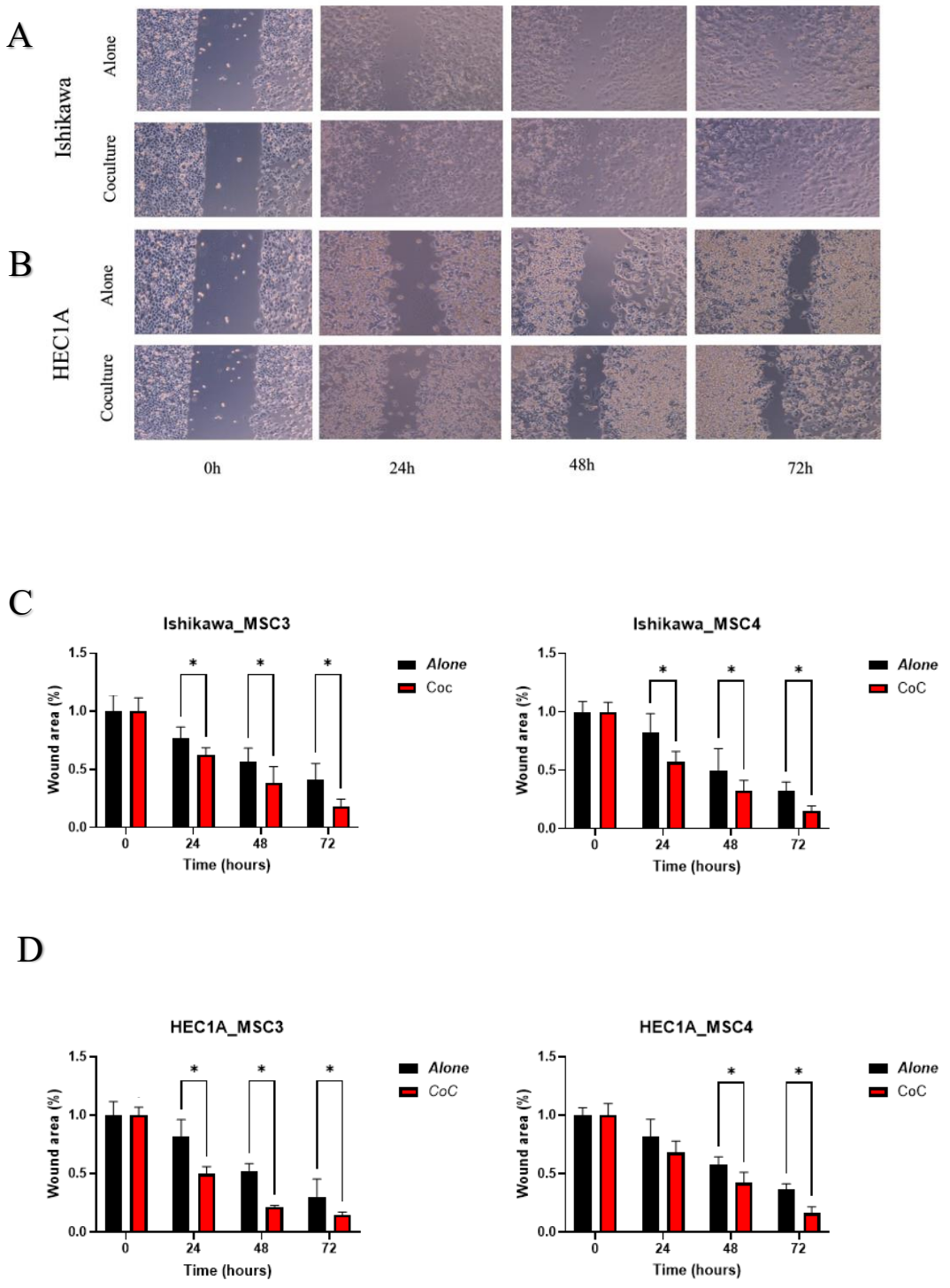

Figure 7 Migration is enhanced in Ishikawa and HEC1A cell lines influenced by AD-MSCs. *Sequential images describing the migration of Ishikawa (**A**) and HEC1A (**B**) cells during the wound healing process at, 0-, 24-, 48-, and 72-hours post-wounding (cells cultured alone, and co-cultured with MSC3, MSC4). The measurements are normalized to the initial wound area at time zero (*C, D*). Data represent the mean ± SD of at least 3 independent experiments performed in triplicate. Statistical significance is indicated against the control group (**Alone**). Student's t-test: *p<0.005.*

5.3 EC cells exhibit enhanced clonogenic ability when co-cultured with AD-MSCs

The capacity for rapid and uncontrolled growth, the development of resistance to chemotherapy treatments, and the potential to invade and spread to distant tissues and organs are features of cancerous cells, making cancer treatment complex and challenging. [80].

To fully characterize EC cells' behaviour when cocultured with AD-MSCs we evaluated the ability of EC cells to form colonies, through a 2D colony formation assay (**Figure 8 A**). The single-cell growth ability to form colonies was evaluated under two conditions: cells grown alone (***Alone***) and cells co-cultured with AD-MSCs (*Coc*). The cell lines Ishikawa and HEC1A, when grown in isolation, displayed a baseline colony-forming capacity (**Figure 8 B-D**). However, after the coculture with AD-MSCs, there was a notable shift. Specifically, in cocultured cells there was an increase in the number of colonies, suggesting that the solitary cancer cells could form colonies on their own, thus exhibiting stronger clonogenic potential when influenced by AD-MSCs. Our data suggest an interactive dynamic where the AD-MSCs favour the clonogenic ability of cancer cells, suggesting that AD-MSCs are likely to support tumour growth and development.

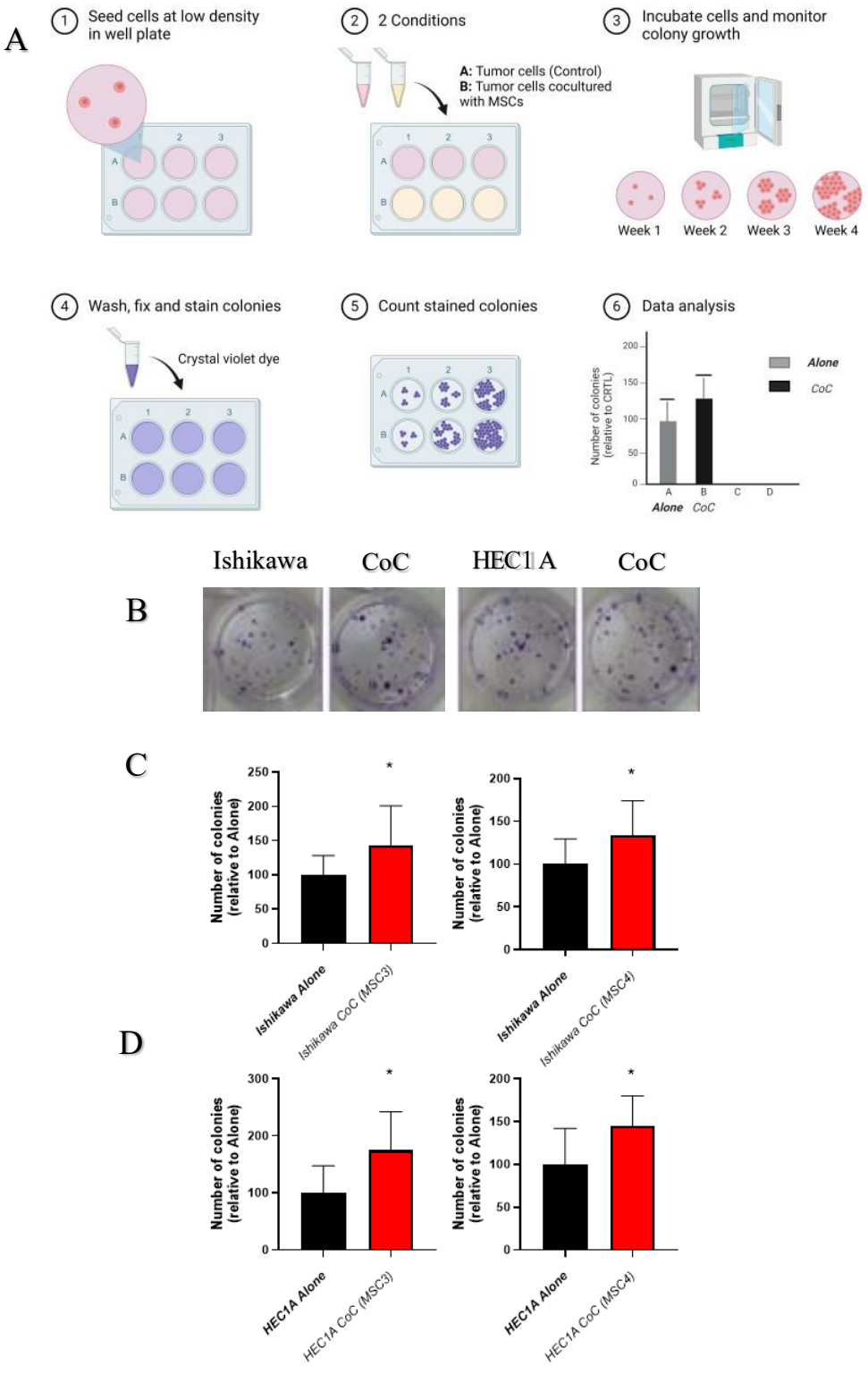

Figure 8 Co-cultured with MSCs enhances the clonogenic ability of EC cells. *Scheme of the experiment (**A**). Cells are seeded at low density in a 24-well plate and grown under two conditions: control (**Alone**) and co-culture with MSCs (Coc). After incubation, colony growth was monitored over four weeks. Representative picture of colony formation (*B*). Quantitative analysis of colony formation in Ishikawa (**C**) and HEC1A (**D**) cell lines. The results are expressed as the number of colonies relative to the control (**Alone**). Data represent the mean +. SD of the experiment performed in triplicate. Student's t-test: *, p<0.05.*

5.4 AD-MSC and EC cells crosstalk results in enhanced intracellular lipid levels

Dysregulated lipid metabolism, a hallmark of cancer, promotes various aspects of tumour biology including proliferation, invasion, and metastasis. [61]. Based on these studies, we cocultured cells for 14 days and stained them with the intracellular Nile red dye. Our analysis revealed a significant increase in both acidic and neutral lipids within AD-MSCs when cocultured with EC cell lines (**Figure 9**). This indicates that there was an important influence of EC cell-secreted factors on AD-MSCs lipid metabolism. Interestingly, a similar increase in lipids metabolism was also observed within EC cell lines. Particularly, when Ishikawa and HEC1A cell lines were cocultured with MSC3, a significant accumulation of neutral lipids was observed, accompanied by slight changes in acidic lipids as illustrated in **Figure 9 B**, **G**. Moreover, it was observed that the Ishikawa cell line exhibited stronger Nile red quantification compared to the HEC1A cell line when cocultured with MSC4, as indicated in **Figure 9 D, E, I** and, **J**. Our data suggest that the effect of crosstalk may depend on the specific tumour type and the type of AD-MSCs involved. These findings underline a metabolic relationship between AD-MSCs and EC cells when they are close, leading to the accumulation of lipid in both cell lines.

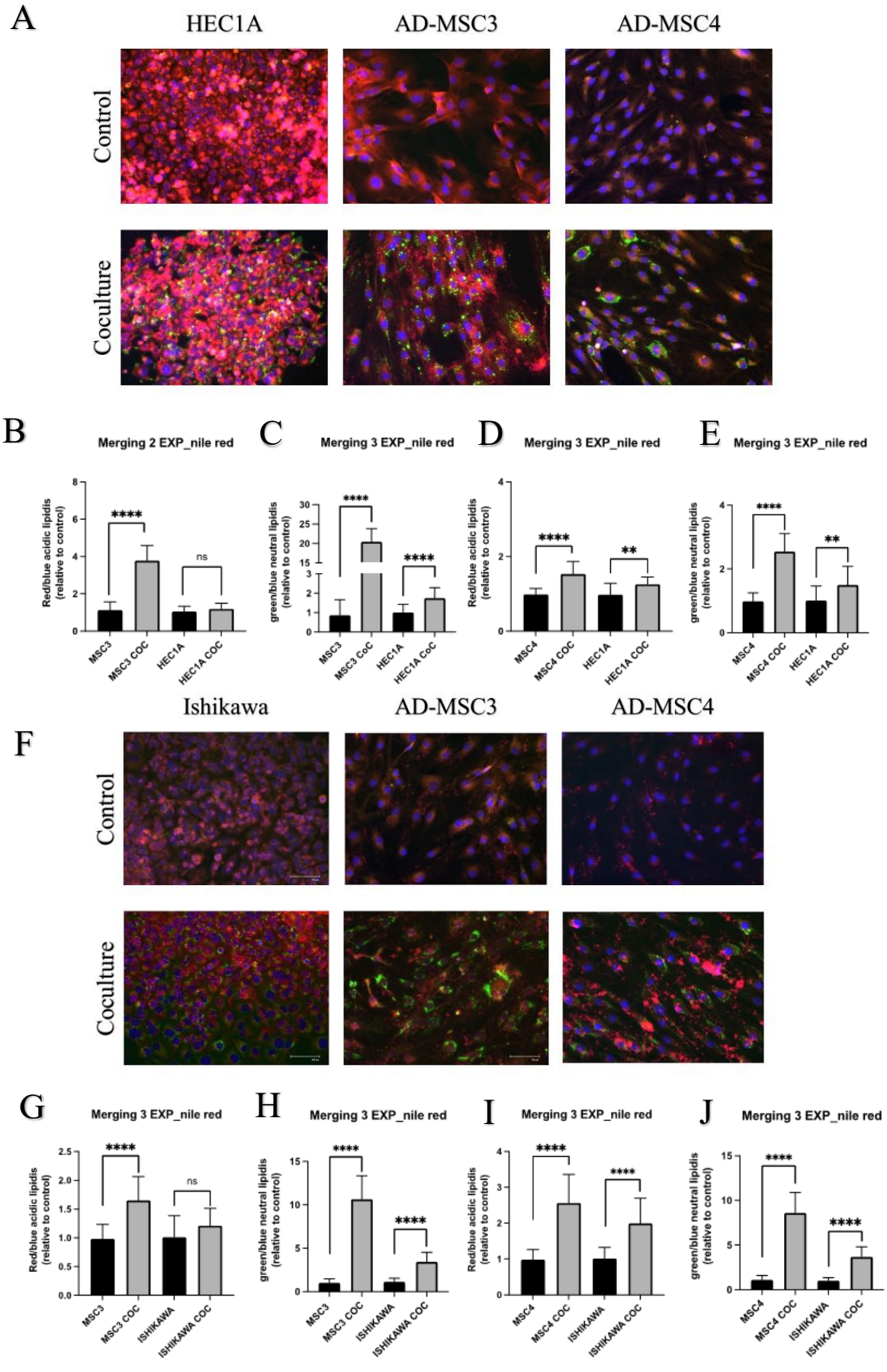
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Figure 9 Lipid Accumulation in cocultured AD-MSCs and EC Cells. *AD-MSCs and EC cells in a 14-day coculture revealed lipid increases, assessed with Nile red dye for lipid content. Fluorescence microscopy captured the lipid distribution at specific light intensities: blue 10%, red 40%, and green 30% for respective visualizations (panels A, F). Nile red quantification indicated: red for acidic lipids, green for neutral lipids, and blue for nuclei. The analysis included Ishikawa cells with AD-MSC3 and AD-MSC4 (panels B-E) and HEC1A cells with AD-MSC3 and AD-MSC4 (panels G-J). Data represent the mean ± SD of at least 3 independent experiments performed in triplicate. Student's t-test: *, p<0.05; **, p<0.01 ***, p<0.001 ****, p<0.0001.*

5.5 Enhancement of mitochondrial function in cocultured EC cells

It is known that the accumulation of lipids in cancer cells can lead to significant metabolic alterations which may promote tumorigenesis and cancer progression [72]. Thus, we investigated the impact of AD-MSCs on mitochondrial activity in cancer cell lines starting from the mitochondrial membrane potential, a vital indicator of cellular metabolism and energy production [82], which is influenced by lipid accumulation. To this end, we utilized JC-1 in EC cells control (alone) and co-cultured with AD-MSCs (**Figure 10 A**). We evaluated the red/green fluorescence ratio of JC1 dye, the staining differentiates JC-1 aggregates, indicative of high membrane potential, and JC-1 monomers, representative of low membrane potential often associated with apoptosis or metabolic stress. EC cells cocultured with AD-MSCs (*CoC*) displayed a significant increase in the red/green fluorescence ratio (**Figure 10 B-D**), especially in the HEC1A cells, compared to control cells (***Alone***), (**Figure 10 B, D**). Altogether, our data suggests that AD-MSCs, in both cell lines, may help to stabilize or enhance the mitochondrial membrane potential.

From these observations, we suppose that AD-MSCs could promote lipid synthesis or absorption in cancer cells, leading to an enhanced rate of mitochondrial respiration and biogenesis in these cells, potentially impacting cancer cell metabolism and growth.

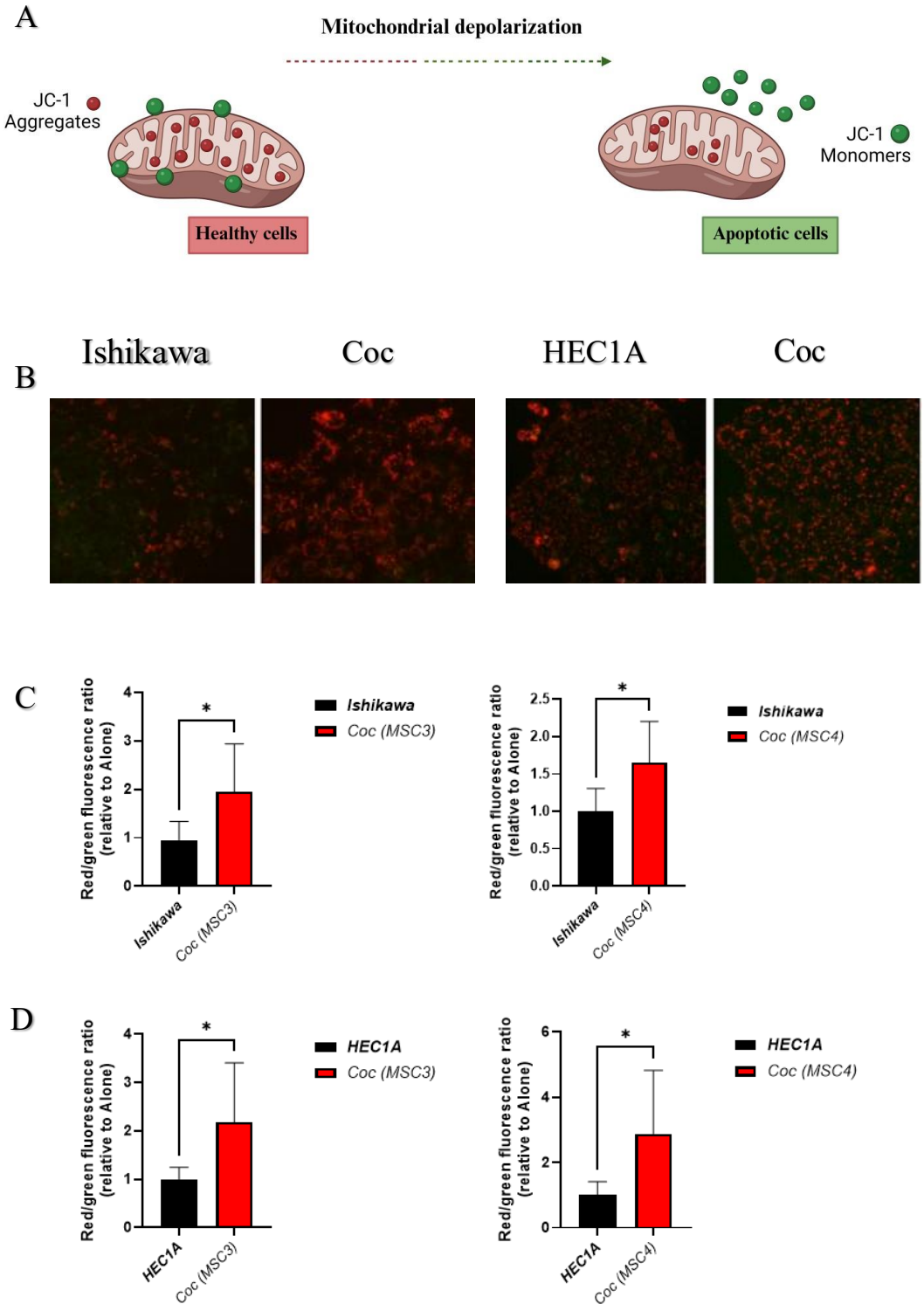

Figure 10 EC cells show mitochondrial membrane hyperpolarisation after coculture with AD-MSCs *Schematic representation of JC1 assay (**A**): JC-1 aggregates, indicative of healthy cells with high mitochondrial membrane potential, and JC-1 monomers, indicative of apoptotic cells with low mitochondrial membrane potential. Representative pictures of Ishikawa and HEC1A cells stained with JC1 dye* (B). *Bar graphs representing the red/green fluorescence ratio for EC cell line alone and coculture: Ishikawa cells* (C) *and HEC1A cells (**D**); Data represent the mean ± SD of the experiment performed in triplicate; Student's t-test: *, $p<0.05$.*

6. DISCUSSION

Cancer development is stimulated by genetic components, environmental exposures, and lifestyle exposures, all impacting the cellular microenvironment and initiating carcinogenesis [83]. Among lifestyle factors, obesity, now recognized as a global epidemic, is linked to an increased risk of certain cancers, especially those influenced by hormones disequilibrium such as cancers of the endometrium, breast, ovaries, and prostate [83]. The connection between obesity and these cancers is largely attributed to how obesity and excessive nutritional intake disrupt the body's internal hormonal balance and alter the cellular microenvironment. These alterations can create conditions that favour the initiation and progression of cancer and may also impact the effectiveness of cancer treatments [84]. Specifically, obesity can lead to elevated levels of certain hormones, including estrogen and insulin, which can stimulate the growth and spread of cancer cells [53]. This hormonal imbalance underscores the critical link between obesity and increased risk of developing cancer. [84]. EC, the most common gynaecological malignancy, was one of the first cancers recognized to be associated with elevated BMI, as highlighted in a 2018 study by the American Institute for Cancer Research. The study revealed that for every 5 kg/increase in BMI, the risk of developing EC grows by 50% [28]. Adipose tissue is an active organ that not only stores extra energy, but it functions as an endocrine organ capable of synthesizing several biologically active compounds named adipokines that influence every aspect of energy balance and metabolism regulation [32]. The relationship between tumours and adjacent adipose tissue has recently become a crucial point of research, especially considering the proximity of tumours to adipose tissue, both locally and during metastasis. Adipose tissue is a complex mix of various cell types, including adipocytes, mesenchymal stem cells (MSCs), endothelial cells, fibroblasts, immune cells, and vascular smooth muscle cells. These cells interact directly or indirectly communicating through paracrine factors such as adipokines and cytokines [32]. Among the diverse cell populations within adipose tissue, AD-MSCs have recently come to attention for their significant role in transforming the TME and their significant alterations in the context of obesity [59]. Our research focused on the interactions between visceral AD-MSCs and EC cell lines, investigating *in vitro* the role of these mesenchymal stem cells in facilitating the growth, spread, and invasion of cancer cells [85]. It is known that the composition of the TME is influenced by cells deriving from two primary sources: adjacent local tissues and distant organs like the bone marrow, which send cells through the circulatory system[55]. The specific types and origins of cells within the TME can differ significantly across various tumours and remain unknown. Typically, tumours attract cells from nearby tissues, which are abundant in fibroblasts, pericytes, and vascular cells, all crucial for the healthy functioning of tissues. While the origins of MSCs within tumours are still being unexplored, there is growing evidence that demonstrates their derivation from either the bone marrow or, according to newer

research, directly from adipose tissue [73].Given its high concentration of MSCs and its proximity to many tumour locations, visceral adipose tissue is increasingly recognized as a key potential MSC reservoir for cancer development [85] Herein, with a 2D coculture system, we explored how AD-MSCs interact with EC cells. Particularly, in our assay, AD-MSC enhanced the growth of EC cells through paracrine signalling. . Our data align with findings by Sarhadi et al., 2021 which examines osteosarcoma (OS), a severe bone cancer, known for its high risk of recurrence and spread. It suggests OS may originate from MSCs differentiation. MSCs in the tumor environment interact with OS cells, affecting their growth and spread through signalling molecules. Particularly, extracellular vesicles (EVs) from MSCs and OS cells facilitate this communication by transferring genetic material and proteins [86]. More recently research conducted by Slama Y. et al., 2023 highlights the MSCs' role in tumor progression. These cells are known to interact with the TME, modulate tumor behaviour, influence their functions, and promote distant metastasis through the secretion of mediators, the regulation of cell-cell interactions, and the modulation of the immune response [87].A study conducted in 2017 by Hill BS. et al. revealed that MSCs derived from bone marrow and adipose tissue can be recruited to the TME in response to multiple signals: chemokines, cytokines, and growth factors. When recruited, they evolve and differentiate in tumor-associated MSCs and cancer-associated fibroblasts resulting in the sustenance of proliferative signalling, cell death mechanism evasion, angiogenesis, and cancer spreading as well as invasion[88].

Moreover, a study by So et al, 2015 has shown that MSCs enhance EC metastasis by triggering the Epithelial-Mesenchymal Transition, a key step for tumour spread and invasion [89]. In order to investigate if a similar effect was visible *in vitro* in our system, we performed cell migration assay, and we observed that EC cells exhibit significantly higher migratory capabilities when co-cultured with AD-MSCs compared to EC cells cultured alone, suggesting that the interaction with AD-MSCs within TME may sustain the invasive potential of EC cells. These results are crucial for future investigations aiming at understanding the metastatic behaviour and invasive capacity of EC cells in obese women [90].

Another crucial characteristic of tumour progression is the ability of cancer cells to counteract anoikis, a form of programmed cell death induced by the detachment of cells from their extracellular matrix or neighboring cells, preventing their survival in inappropriate locations [91]. Recently, several studies have shown that cancer cells can exhibit enhanced colony formation, a read out of anoikis resistance, when cocultured with MSCs. This phenomenon was highlighted in a study conducted by Oh et al. in 2020, which observed that when colorectal cancer cells (CRC) were co-cultured with MSCs derived from bone marrow and adipose tissue, there was an evident increase in colony formation compared to control cells. This study also indicated that cocultured CRC

displayed enhanced invasive and proliferative capacities of CRC cells through alteration of p53 and TGF-β1 levels [92]. Based on these findings, we performed similar experiments on EC cells cocultured with AD-MSCs. Our data confirmed that EC cells show significantly higher number of colonies when co-cultured with AD-MSCs suggesting that AD-MSCs support effect on the clonogenic ability of the cancer cells. Since, enhanced clonogenic growth in the presence of AD-MSCs could imply that EC cells have an increased potential for metastasis, as colony formation is often associated with the ability of cancer cells to detach, survive in circulation, and establish new tumours in distant organs [93], further experiments should be conducted to confirm in 3D model an *in vivo* our data.

Also, chemoresistance usually occurs during cancer progression [94], thus we examined EC cells response to a well-known chemotherapeutic cytotoxic and apoptosis-inducing agent, paclitaxel [95]. Our experiments showed that both EC cells grown in a co-culture developed increased resistance to chemotherapy compared to control cells. Our data suggests that AD-MSCs support the proliferation of EC cells highlighting that the presence of AD-MSCs in the TME and their interaction is complex, as they can alter the behavior of cancer cells. Notably, previous works reported that MSCs are likely to increase chemoresistance to paclitaxel by secreting growth factors and cytokines, such as IL-7, IL-8, IGF, and EGF [55]. These factors can promote survival pathways within cancer cells, making them less susceptible to the cytotoxic effects of chemotherapy. Notably, in a more complex model, MSCs have been shown to inhibit the anti-tumour immune response, including innate and adaptive immune responses, by secreting in the TME a variety of soluble factors and mediators such as PGE2, IFNγ, IL-4, TGF-β1, and IL-6 resulting in a promoted tumour evasion and growth [55]. Further studies are needed to elucidate if, even in our experiment model, AD-MSCs secrete soluble factors, including cytokines, chemokines, and growth factors, which can contribute to creating a pro-tumorigenic environment. Within this context, coculture experiments will be conducted on 3D models to explore AD-MSCs activity on EC cells as well as on immune cells, such as T cells and natural killer (NK) cells, which are critical for the anti-tumour immune response [96].

Moreover, in the context of chemotherapy resistance, AD-MSCs can induce the expression of drug-resistance genes, activate anti-apoptotic pathways, and enhance DNA repair mechanisms in cancer cells. This can make it more difficult for chemotherapeutic agents to kill cancer cells, leading to treatment failure and disease progression [97]. The implications of these findings are significant for cancer therapy. Targeting the interaction between AD-MSCs and cancer cells, or the factors released by AD-MSCs, could potentially improve the efficacy of cancer treatments, including chemotherapy. Further studies are needed to develop strategies to either inhibit the supportive role of AD-MSCs in

cancer progression or to reprogram them to have a tumour-suppressing function to treat EC and possibly other malignancies.

It is well established that EC is closely related to fat content, and dyslipidemia is among the most significant metabolic changes in this cancer [98]. Also, cancer cells often undergo metabolic reprogramming to support rapid proliferation and survival under various conditions, such as hypoxia and low nutrient levels [99]. Considering the link between lipids and mitochondrial activity; in our study, we observed that EC cells cocultured with AD-MSCs exhibited a significant increase in mitochondrial membrane potential and enhanced lipid accumulation within the cocultured EC cells. This indicates a profound metabolic interplay between AD-MSCs and EC cells, which is characterized by a mutual increase in lipid levels. Mitochondrial hyperpolarization can be associated with metabolic reprogramming in cancer cells. this reprogramming often involves increased glycolysis (the Warburg effect) and changes in lipid metabolism, which are adaptations to support rapid cell growth and survival under the different conditions of the TME [62]. This hyperpolarization, studied in certain cancers such as breast cancer, has been linked to a more aggressive cancer phenotype, characterized by a reduced response to chemotherapy, increased secretion of angiogenic factors like VEGF, and greater invasive capabilities [77]. Our preliminary data on mitochondrial hyperpolarization suggest that EC cells may be adapting their metabolic processes, which is a hallmark of cell reprogramming during cell interaction [60]. Indeed, mitochondria are central to both energy production and lipid metabolism [100]. Future research will be needed to identify the specific metabolic changes induced by the coculture and to understand their implications for cancer progression and the development of potential therapeutic targets.

In conclusion, our study highlights the role of visceral AD-MSCs in promoting EC cells growth, chemoresistance, and migration further supporting their role in obesity-related EC. The interaction between AD-MSCs and EC cells suggests a metabolic interaction that could increase EC risk in individuals with excess visceral fat, indicating a need for further research to develop new EC screening, diagnosis, and treatment methods.

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