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Review Article

Anti-Tumor Potential of Mesenchymal Stem Cells (MSCs) Versus 5-Fluorouracil in HT-29 and HCT-116 Colorectal Cancer Cell Lines

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Abstract

Despite the well-established association between Mesenchymal Stem Cells (MSCs) and tumor development, the lack of precise methods to distinguish between different MSC populations, along with their paradoxical roles in tumor progression and suppression, has hindered the advancement of MSC-based oncological therapies. In this study, we investigate the effects of human umbilical cord-derived MSCs (UC-MSCs) and their secretome, both alone and in comparison, with 5-Fluorouracil (5-FU), on the proliferation, migration capacity and tumor marker expression of Colorectal Cancer (CRC) cell lines. Chemotherapy regimens including 5-FU remain the gold standard for CRC treatment; however, their clinical efficacy is often limited by adverse side effects and the development of chemoresistance. In contrast, MSCs derived from Umbilical Cord (UC-MSCs), Bone Marrow (BM-MSCs) and Adipose Tissue (AT-MSCs), along with their bioactive secretome composed of immunomodulatory cytokines and chemokines, have demonstrated promising therapeutic potential in immune and inflammatory diseases in clinical trials. Owing to their natural tropism toward inflammatory signals, MSCs have also been explored as vehicles for targeted drug delivery to tumor sites. In this study, colorectal cancer tumor marker proteins were quantified using ELISA. The proliferative, apoptotic and anti-inflammatory effects of MSCs were assessed by Flow Cytometry (FCM), while Matrix Metalloproteinase (MMP) expression was analyzed using RT-PCR.

Keywords: Mesenchymal Stem Cells; 5-FU; Secretome; Cancer Therapy; Stem Cell Therapy

Abbreviations

MSC: Mesenchymal Stem Cell; 5-FU: 5-Fluorouracil; CRC: Colorectal Cancer; UC-MSC: Umbilical Cord-Derived Mesenchymal Stem Cell; BM-MSC: Bone Marrow-Derived Mesenchymal Stem Cell; AT-MSC: Adipose Tissue-Derived Mesenchymal Stem Cell; MMP: Matrix Metalloproteinase; TIMP: Tissue Inhibitor Of Metalloproteinases; mRNA: Messenger Ribonucleic Acid; RT-PCR: Reverse Transcription Polymerase Chain Reaction; ELISA: Enzyme-Linked Immunosorbent Assay; DNA: Deoxyribonucleic Acid; SVF: Stromal Vascular Fraction; MTT: Thiazolyl Blue Tetrazolium Bromide; CM: Conditioned Media; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; IFN-α: Interferon Alpha; IFN-γ: Interferon Gamma; IL: Interleukin; TNF-

α: Tumor Necrosis Factor Alpha; CA19-9: Cancer Antigen 19-9; CEA: Carcinoembryonic Antigen; AFP: Alpha-Fetoprotein; HLA-DR: Human Leukocyte Antigen DR; CD3: Cluster Of Differentiation 3

Introduction

Colorectal Cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related death worldwide, accounting for approximately 1.9 million new cases and more than 900 000 deaths in 2020 [1]. The global burden of CRC is expected to increase by nearly 60%, reaching over 2.2 million new cases and 1.1 million deaths annually by 2030 [2]. Mortality associated with CRC is largely attributed to tumor recurrence and the development of chemoresistance [3]. Notably, recurrence rates remain high, as many patients are diagnosed at advanced stages of the disease [4].

Mesenchymal Stem Cells (MSCs) have emerged as promising candidates in regenerative medicine due to their self-renewal capacity, high proliferative potential and ability to differentiate into mesodermal lineages. In addition to these properties, MSCs exert significant paracrine and trophic effects, secreting a wide range of bioactive molecules that promote tissue repair and modulate immune responses [5].

The therapeutic potential of MSCs has been investigated in numerous phase I, II and III clinical trials, primarily focusing on conditions such as graft-versus-host disease and the enhancement of hematopoietic stem cell engraftment [6]. However, their application in oncology remains limited, with only a small number of trials exploring MSC-based therapies for cancers, including gastrointestinal, lung and ovarian malignancies [6]. In the context of CRC, studies have mainly utilized bone marrow-derived MSCs engineered to deliver therapeutic cytokines [6].

Bone Marrow (BM) was the first identified source of MSCs for cell-based therapies, followed by Adipose Tissue (AT) and, more recently, Umbilical Cord (UC) tissue. Compared to BM-derived MSCs, AT- and UC-derived MSCs offer practical advantages, including less invasive collection methods and higher cell yield. Adipose tissue-derived MSCs can be readily isolated from lipoaspirates obtained during cosmetic procedures and expanded efficiently under standard culture conditions. Meanwhile, UC-derived MSCs exhibit superior proliferative capacity and extended culture longevity compared to other sources [7].

Despite these advances, no MSC-based therapy has yet demonstrated definitive curative efficacy for CRC. Therefore, this study aims to investigate the anti-tumor potential of MSCs derived from Adipose Tissue (AT), Bone Marrow (BM) and Umbilical Cord (UC) and to evaluate their comparative effects as potential therapeutic agents against colorectal cancer.

Materials and Methods

Collection of MSCs

A total of ten collections were performed from each source of Mesenchymal Stem Cells (MSCs), including Bone Marrow (BM), Umbilical Cord (UC) and Adipose Tissue (AT). Bone marrow aspirates were obtained by puncturing the iliac crest of participants aged between 30 and 60 years at the hematology department of the Middle East Institute of Health University Hospital. Umbilical cord units were collected from the placenta of full-term deliveries using a multiple bag system containing 17 mL of citrate phosphate dextrose buffer (Cord Blood Collection System; Eltest, Bonn, Germany) and processed within 24 hr of collection. AT was obtained from participants aged between 26 and 57 years, who underwent liposuction at Hotel Dieu de France Hospital (HDF) (Beirut, Lebanon). The Saint-Joseph University and the HDF Ethics Review Board approved the retrieval of all MSC collections (approval reference number: CEHDF1142) and all patients were asked to read and approve/sign informed consent forms prior to any participation.

Isolation and Culture of MSC from Bone Marrow

Bone marrow aspirates were collected by puncturing the iliac crest of participants aged between 30 and 60 years. The aspirates were diluted in a 1:5 ratio with 2 mM Ethylenediaminetetraacetic Acid (EDTA) in Phosphate-Buffered Saline (PBS) (Sigma-Aldrich). The Mononuclear Cell (MNC) fraction was isolated through density gradient centrifugation at 435 g for 30 minutes at room temperature using Ficoll Hypaque-Plus solution (GE Healthcare BioSciences Corp). These cells were then seeded at a density of 1×10^6 cells per cm^2 into T75 or T175 cell culture flasks (Sigma-Aldrich). The first medium change was performed within three days of isolation. The resulting fibroblast-like adherent cells were referred to as Bone Marrow-derived fibroblast-like adherent cells (BM-MSCs) and were cultured at 37 °C in a humidified environment with 5% CO_2. The expansion medium

was composed of Dulbecco's modified Eagle's medium-alpha modification (Alpha-MEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 5% penicillin-streptomycin-amphotericin B solution (PSA: Hyclone; GE Healthcare, Logan, UT, USA). BM-MSCs were maintained in Alpha-MEM with 10% FBS and 5% PSA until they achieved 70 to 90% confluency. Cells were harvested at subconfluence using Trypsin (Sigma-Aldrich) and were replated at an average density of $1.3 \pm 0.7 \times 10^3/cm^2$ from the second passage onward.

Isolation and Culture of MSC from Human Umbilical Cord Wharton Jelly

Umbilical cord samples were collected in Phosphate-Buffered Saline (PBS) supplemented with 10% penicillin-streptomycin-amphotericin B (PSA) and transported to the laboratory within a maximum of 12 hours. After washing, the cord samples were cut into 1-2 cm sections and the umbilical vessels (both arteries and veins) were removed. Wharton's jelly was then collected, minced into small pieces and digested with collagenase overnight before being cultured in flasks. Non-adherent cells were discarded 12 hours after the initial plating. The same culture conditions and media used for Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs) were applied. Adherent fibroblast-like cells, referred to as UC-MSCs, formed colony-forming units (CFU-F) and were harvested at subconfluence using Trypsin (Sigma-Aldrich). From the second passage onward, these cells were replated at an average density of $3.5 \pm 4.8 \times 10^3/cm^2$.

Isolation and Culture of MSC from Adipose Tissue

Adipose tissue was collected from participants aged between 26 and 57 years who underwent liposuction. To isolate the Stromal Vascular Fraction (SVF), the lipoaspirates were thoroughly washed with Phosphate-Buffered Saline (PBS) containing 5% Penicillin-Streptomycin-Amphotericin B (PSA). The lipoaspirates were then digested with an equal volume of 0.075% collagenase type I (Sigma-Aldrich) for 30 to 60 minutes at 37 °C with gentle agitation. The collagenase activity was neutralized using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). To obtain a high-density SVF pellet, the digested lipoaspirate was centrifuged at 1,200 g for 10 minutes. The pellet was resuspended in DMEM containing 10% FBS and filtered through a 100 μm nylon cell strainer (Falcon). The filtered cells were then centrifuged again at 1,200 g for 10 minutes. The resuspended SVF cells were plated at a density of 1 × 10^6/cm² into T75 or T175 culture flasks. Non-adherent cells were removed 12 to 18 hours after initial plating by thoroughly washing the plates. The resulting fibroblast-like adherent cells, known as AT-derived fibroblast-like adherent cells, were cultivated under the same conditions as the Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs). Adipose Tissue-derived MSCs (AT-MSCs) were harvested at subconfluence using Trypsin (Sigma-Aldrich) and were replated from the second passage onward at an average density of $1.8 \pm 3.1 \times 10^3/cm^2$.

HCT116 and HT29 Cancer Cell Lines Culture

Human colorectal cancer cell lines HCT116 and HT29 were cultured in DMEM/F12 supplemented with 10% FBS supplemented with 5% PSA. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Conditioned Media (CM) Preparation

All MSCs were cultured with DMEM/F12 supplemented with 5% PSA at subconfluency. Thereafter, the supernatant, containing all released cytokines and chemokines to be studied, was collected.

Co-culture Maintenance

Only AT-MSC, BM-MSC and UCMSC prior to passage 3 were used for co-culture experiments. HCT116 and HT29 cell lines were cultured in direct contact with UC-MSCs in DMEM/F12 supplemented with 5% Penicillin-Streptomycin-Amphotericin B (PSA) in a sterile humidified incubator with 5% CO_2 at 37 °C for 48 hours, along with their supernatant under the same conditions. The co-culture was set at a 1:1 ratio. The cell lines served as the control group and were maintained under the same culture conditions and duration.

Assessing Differentiation Potential of MSCs

The cultured cells were differentiated into osteogenic, adipogenic and chondrogenic lineage by culturing in osteogenic medium [DMEM supplemented with 10- 8 M dexamethasone (Sigma, D4902), 10 mM β glycerophosphate (Sigma, G9422) and 50 μg/ml ascorbic acid], adipogenic medium [DMEM supplemented with 10 mM 3 isobutyl-1-methylxanthine (Sigma, 17018), 0.1 mM indomethacin (Sigma, 17378), 10 μg/ml insulin (Sigma, I6634), 10- 6 dexamethasone] and chondrogenic medium (Stempro,

Invitrogen) and confirmed by staining with Alizarin red (Sigma, A5533), Oil red O (Sigma, O0625) and Alcian blue (Himedia Laboratories, Mumbai, India: Cat no RM471-1 g staining, respectively).

Flow Cytometry Analysis

For surface marker immunophenotyping, cells were stained with the following conjugated antibodies: anti-CD45-vioblue, anti-CD34-PE, HLADr-vioblue, anti-CD73-PE, anti-CD90-FITC, anti-CD105-vioblue, CD24-PE CD44-FITC, CD133-APC, CD14-PE and relevant isotypes (Miltenyi-Biotec). We acquired at least 20,000 events as test samples.

Cytokine Analysis

For cytokine analysis, we used the MACSplex Cytokine 12 kit. Supernatants were combined with specific capture beads for each cytokine, including Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF), Interferon (IFN)-α, IFN-γ, Interleukin (IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A and Tumor Necrosis Factor (TNF)-α. Antibodies conjugated with PE were then added and incubated for 2 hours at room temperature, protected from light. After centrifugation, the pellet containing the beads was resuspended. Flow cytometric acquisition and data analysis were conducted using the MACSQuant® express mode. Background signals were evaluated by analyzing beads incubated solely with cell culture medium and these signals were subsequently subtracted from those of the beads incubated with the supernatants.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from samples using the QIAamp RNA extraction kit (Qiagen Inc., Valencia, CA, USA), selected for its high purity and reliability in isolating intact RNA. The quality and yield of RNA were assessed using a NanoDrop spectrophotometer to ensure suitability for downstream applications. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA in a 20µL reaction mixture with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, CA), as this input amount provides optimal reverse transcription efficiency.

Quantitative real-time PCR was conducted using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, CA) in triplicate to ensure reproducibility and accuracy of results. The reaction conditions included polymerase activation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds and annealing and extension at 62°C for 20 seconds, which are standard conditions optimized for primer specificity and amplification efficiency. Gene expression levels were normalized to endogenous control genes to account for sample-to-sample variation in RNA quantity and reverse transcription efficiency.

Apoptosis Test

Apoptosis was assessed by staining cell cultures with 10 µL of Annexin V (fluorescein isothiocyanate; Miltenyi Biotec Annexin V-FITC kit) at 4°C for 20 minutes in the dark, followed by counterstaining with 5µL of Propidium Iodide (PI) at room temperature for 5 minutes, also in the dark. Detection was carried out using a MACSQuant analyzer, analyzing each sample of 10^4 cells. The calculation of apoptotic cells was performed using the MACSQuant software.

MTT Proliferation Test

Principle: The MTT assay is a colorimetric method used to evaluate cell viability. Thiazolyl Blue Tetrazolium Bromide (MTT) enters the mitochondria, where it is reduced by mitochondrial succinate dehydrogenase in metabolically active, proliferating cells. This reduction converts the yellow MTT salt into dark purple formazan crystals, which are insoluble products formed during the reaction. The formazan crystals are dissolved in a solubilization solution and the results are quantified using an ELISA plate reader at a wavelength of 550 nm. Reagents: Thiazolyl Blue Tetrazolium Bromide (MTT powder), PBS, isobutanol, 6M HCl, 5-FU, DMSO and SDS. Procedure: Cells were plated in triplicate in flat-bottom 96-well plates at a density of 5,000 cells in 100 µL of media per well. Cells were treated with 40 µM of 5-FU, with MSCs used as a control. After 48 hours, 10µL of MTT dye solution (100 mg of MTT in 20 mL of 1X PBS) was added to each well to achieve a final concentration of 0.5 mg/mL. The cells were then incubated for 3 to 4 hours in a 37 °C, 5% CO_2 incubator. Subsequently, 100 µL of solubilization solution (500µL of 6M HCl, 12.5 mL of isobutanol and 25 g of SDS in 250 mL of distilled water) was added to each well and the cells were incubated overnight at 37°C, 5% CO_2. The optical density was measured using an ELISA plate reader at a wavelength of 550 nm. The percentage of proliferating cells was calculated based on the number of active cells in each condition relative to the total number of control cells and results were expressed as the mean of three independent experiments [8].

Tumor Markers Assay: CA-19-9, AFP and CEA

CA-19-9, AFP and CEA levels were measured using an ELISA. During the process, the supernatant was mixed with a monoclonal biotinylated antibody, forming a complex through the interaction of biotin and streptavidin during a 60-minute incubation. Any excess was removed by washing, after which an enzyme-antibody complex was added to create the final sandwich complex. Following another incubation period, the excess was washed away once more. Sulfuric acid was then added to stop the reaction, resulting in a color change from blue to yellow. The intensity of the yellow color was directly proportional to the concentration of the samples and the absorbance was measured at 450 nm.

Statistical Analysis

Statistical significance was determined based on a paired T-test and one-way ANOVA test. P-values below 0.05 were considered as statistically significant.

Results

Immunophenotyping and Characterization of Mesenchymal Stem Cells (MSCs), Cancer Cell Lines and Their Co-Culture

The characterization of the MSCs surface protein expressions were examined by flow cytometry Fig. 1. All MSCs derived from the different sources expressed CD44, CD73, CD105 and CD133 and were negative for CD45, CD34 and HLADr. BM- and AT-MSCs exhibited higher expression levels of CD105 compared to UC-MSCs, while CD73 showed the lowest expression among all derived MSCs. Moreover, the flow cytometry analysis of the cancer cell line, HT-29, showed that CD44 and (Fig. 1) significantly increase when compared to AT- and BM MSCs and CD44 significantly increased compared to UC-MSCs (Fig. 1) whereas CD133 expression did not changed in HT-29 when compared to UC-MSCs. As for CD105, the expression level decreased in these cancer cell lines when compared to all MSCs. However, in the case of CD73, its expression decreased significantly when compared to AC-MSCs (Fig. 1) and increased significantly compared to UC-MSCs (Fig. 1). As for the cancer cell line, HCT-116, the analysis showed a significant decreased in CD133 expression and a significant increase in CD44, CD73 and CD105 when compared to all MSCs (Fig. 1).

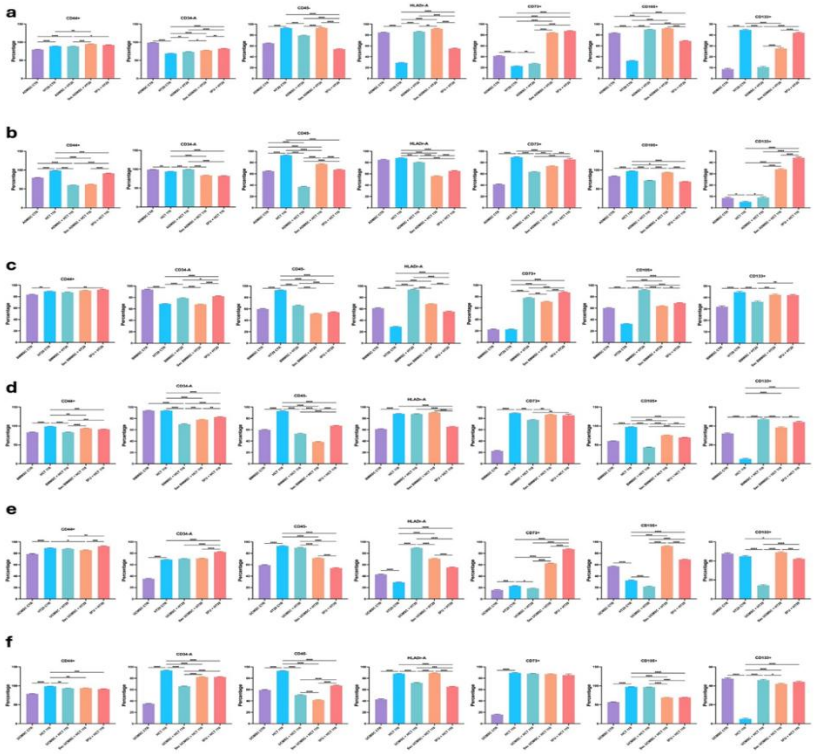

Figure 1: Flow cytometry analysis of MSC markers (CD34, CD44, CD45, CD73, CD105, CD133, HLADr) in adipose-derived (AD) (a-b), bone marrow-derived (BM) (c-d) and cord blood-derived (denoted as UC) (e-f) MSCs, as well as in co-culture with HCT-116 (b, d, f) and HT-29 (a, c, e) cancer cell lines and their secretomes. MSCs were positive for CD44, CD73, CD105 and CD 133 and negative for leukocyte common antigen CD45, the hematopoietic lineage marker CD34 and the macrophage markers HLADr. All data are shown as mean ± SD, n=3, * $p<0.05$, ** $p<0.005$, ***$p<0.001$, ****$p<0.0001$.

In-vitro culture of HCT-116 and HT-29 colorectal cancer cells serves as a simplified model for studying interactions within the Tumor Microenvironment (TME), particularly with T-cells. We examined CD3 expression to understand immune cell infiltration and potential responses within the TME. Both cell lines initially showed high CD3 levels, which decreased upon co-culture with MSCs, their secretome or treatment with 5-FU, as shown in Fig. 2

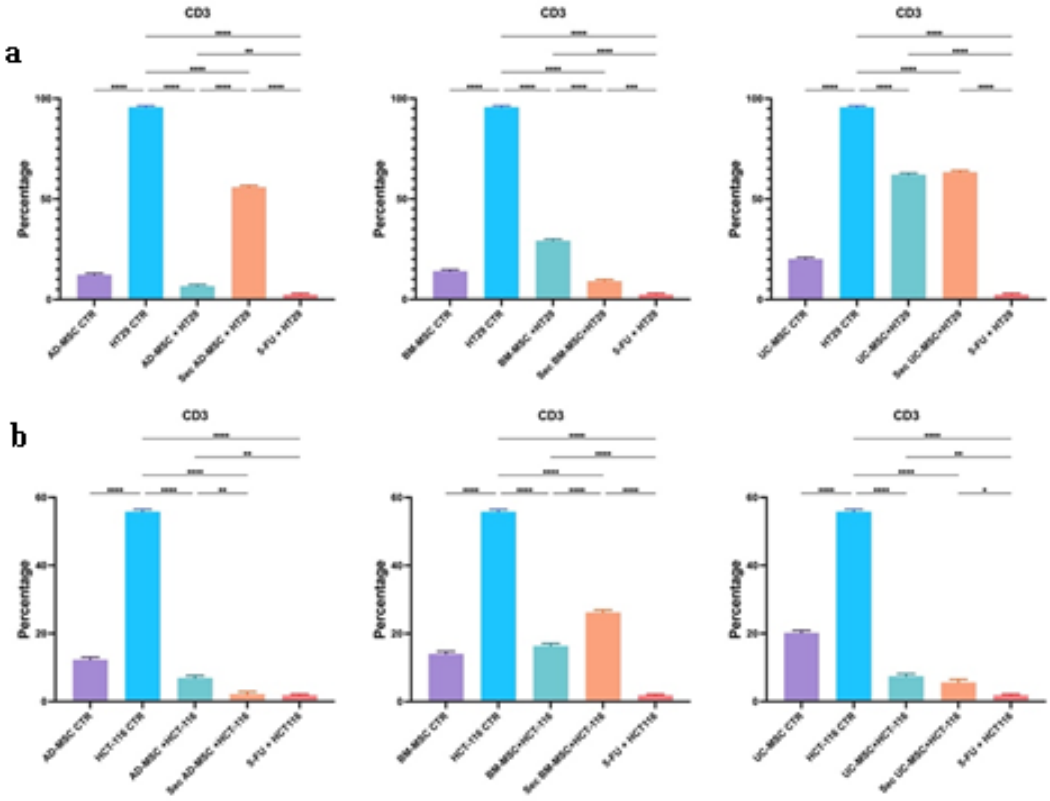

Figure 2: Flow cytometry analysis of the surface marker CD3 in MSCs and their secretome when co-cultured with HT-29 and HCT-116 cancer cell lines. All data are shown as mean ± SD, n=3, * p<0.05, ** p<0.005, ***p<0.001, ****p<0.0001.

Inhibition of Tumor Markers by AT, BM and UC-MSCs and Their Secretomes

To investigate the influence of MSCs on tumor cell markers, flow cytometry analysis was conducted on CD44 (Fig. 1). The results showed a significant decrease in the level of CD44 in HCT-116 cell lines when co-cultured with AT-, BM- and UC-MSCs and their derived secretomes (Fig. 1). As for HT-29, the levels of CD44 did not change when treated with BM- and UC-MSCs (Fig. 1) and their derived secretome and it significantly increased when these cells were treated with AT-MSCs and its secretome (Fig. 1). Moreover, when HCT-116 and HT-29 were treated with 5-FU, the chemotherapeutic drug conventionally used for treating colorectal cancer, the levels of CD44 significantly increased upon treatment.

CEA, AFP and CA-19-9 are established markers for colorectal cancer detection. We conducted ELISA tests to measure their levels when co-cultured with various Mesenchymal Stem Cells (MSCs) and their secretomes, as well as under 5-Fluorouracil (5-FU) treatment. Our results indicate a significant decrease in CEA, AFP and CA-19-9 expression in HT-29 cells when exposed to different MSCs and their secretomes, mirroring the response observed with 5-FU treatment (Fig. 3). Additionally, RT-PCR analysis demonstrated a reduction in AFP gene expression in HT-29 cells when co-cultured with MSCs and their secretomes (Fig. 3), except for an increase in AFP gene expression observed when co-cultured with the secretome derived from BM-MSCs.

In contrast, CEA levels increased in HCT-116 cells when co-cultured with various MSCs and their secretomes, while AFP and CA-19-9 levels decreased under the same conditions (Fig. 3). Notably, AFP gene expression did not decrease in HCT-116 cells when co-cultured with MSCs and their secretomes or when treated with 5-FU (Fig. 3).

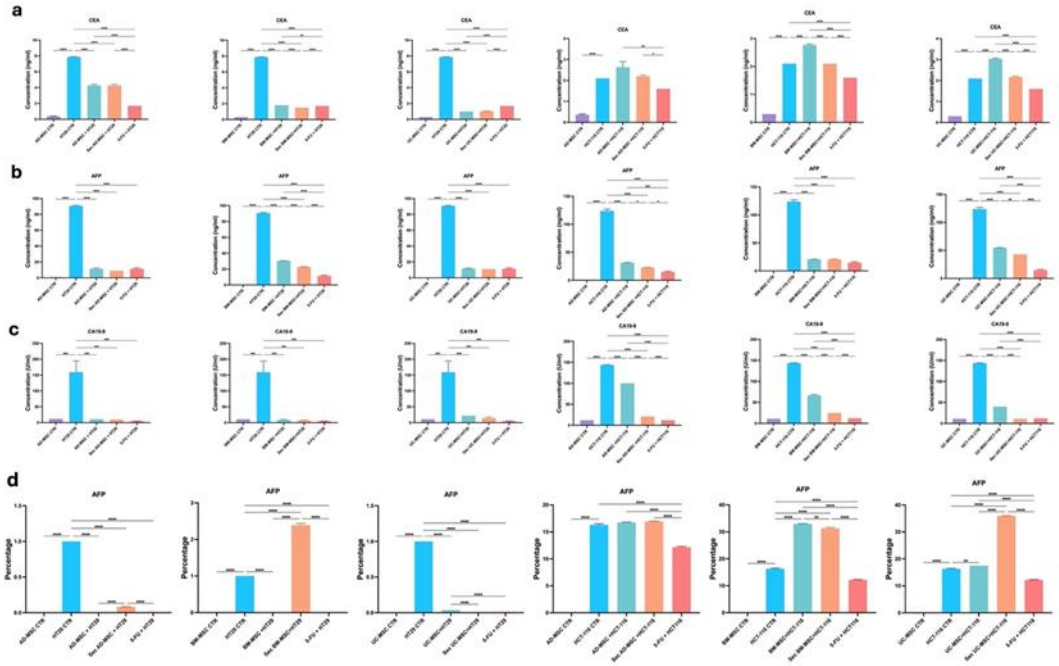

Figure 3: Expression of tumor markers. (a-c) Cell culture supernatant from co-culture of HCT-116 and HT-29 with AD-, BM- and UC-MSCs and their corresponding secretomes was collected and analyzed for detection of CEA, AFP and CA19-9. (d) Real time PCR showing the mRNA levels of AFP in HCT-116 and HT-29 upon treatments with the different type of MSCs and their secretomes. The results for the PCR are represented as percentage of controls. All data are shown as means ± SD, n=3, * p<0.05, ** p<0.005, ***p<0.001, ****p<0.0001.

Cell Proliferation Inhibition and Apoptosis Induction

Cancer cells co-cultured directly with adipose tissue-derived, bone marrow-derived and umbilical cord-derived Mesenchymal Stem Cells (MSCs) exhibited increased apoptosis, cellular death and reduced viability (Fig. 4). Moreover, co-culture with secretomes from AD-, BM- and UC-MSCs respectively resulted in even greater apoptotic activity and decreased viability compared to direct co-culture with MSCs. Interestingly, the effects of secretome treatment were comparable to those observed in cancer cells treated with 5-Fluorouracil (5-FU).

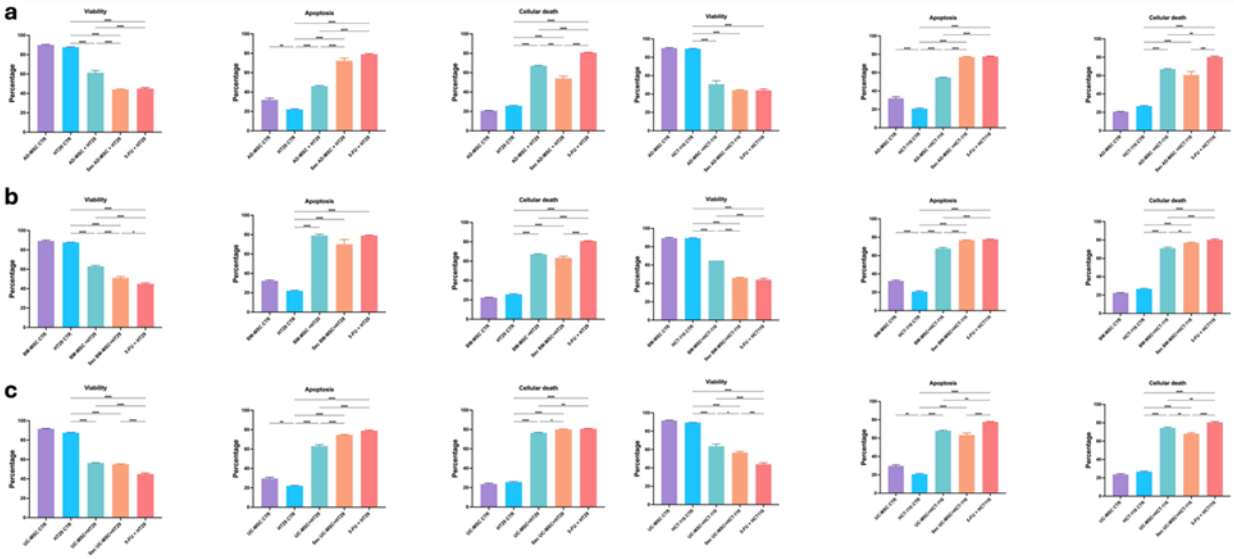

Figure 4: Apoptosis and cell death. a-c Viability essay showed an increase in cell death in cancer cell lines. All data are shown as means ± SD, n=3, * p<0.05, ** p<0.005, ***p<0.001, ****p<0.0001.

Impact of Mesenchymal Stem Cells on Tumor Invasion and Metastasis

The initial regulation of migration and invasion involves the dysregulated expression of Matrix Metalloproteinases (MMPs) and Tissue Inhibitor Metalloproteinases (TIMPs). To assess the potential contribution of TIMPs to the reduced migration and invasion of HT-29 and HCT-116 cells following co-culture with MSCs or their derived secretomes, we examined the expression of TIMP-1, -2 and -3 using real-time PCR. Our findings indicate that BM- and UC-MSCs and their secretome significantly upregulate mRNA levels of TIMP-1, -2 and -3, in the HT-29 cancer cells (Fig. 5). This response was only observed with AT-MSCs but not their secretome (Fig. 5). On the other hand, when HCT-116 was co-culture with MSCs and their secretome, mRNA levels of TIMP-1, -2 and -3 did not change significantly (Fig. 5). As for the metalloproteinases, co-culturing these cancer cells with MSCs upregulated the expression of MMP-2 and MMP-9 mRNA (Fig. 5).

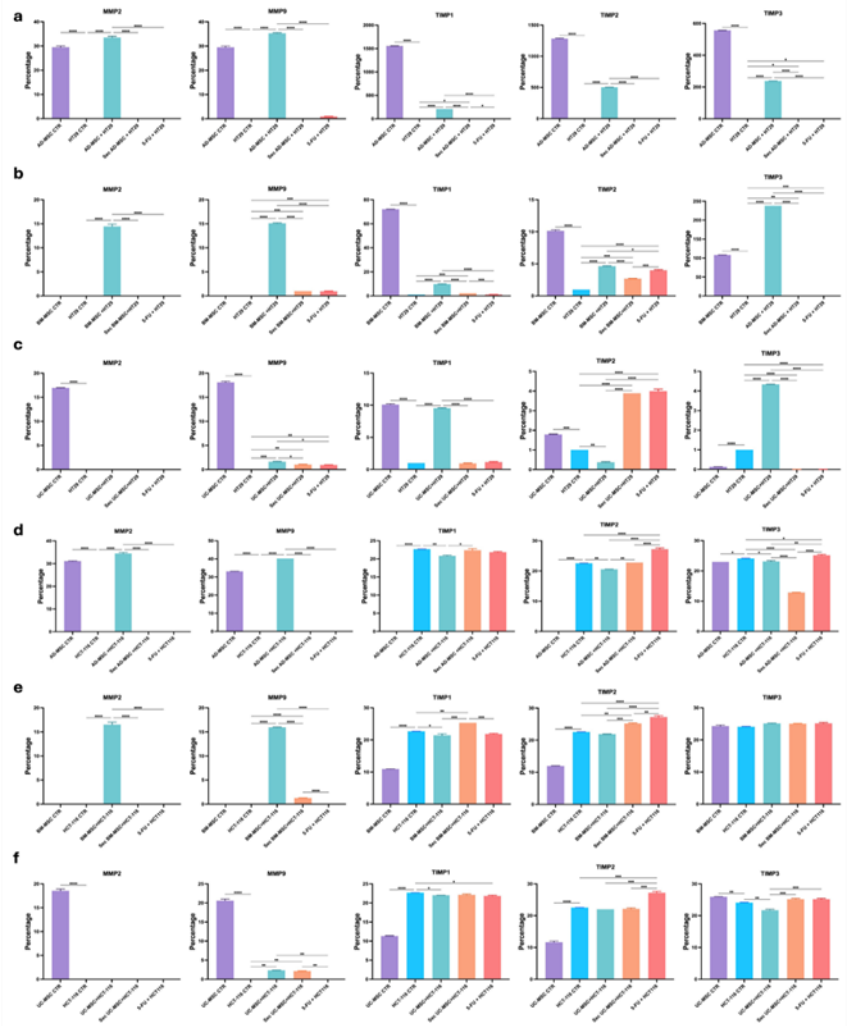

Figure 5: mRNA expression of MMPs and TIMPs. All data are shown as means ± SD, n=3, * p<0.05, ** p<0.005, ***p<0.001, ****p<0.0001.

The Anti-Inflammatory Potential of MSCs and Their Secretomes

Flow cytometry analysis of cytokine profiles in supernatants collected from AT-MSCs, BM-MSCs, UC-MSCs co-cultured with HT-29 and HCT-116 cells, either directly or with their derived secretomes, revealed alterations in the levels of several cytokines. As shown in Fig. 6, HT-29 cells exhibited low levels of the anti-inflammatory cytokines IL-4 and IL-10. However, upon co-culture with MSCs and their secretome, IL-10 levels were increased. Regarding IL-4 levels, we noted a modest increase in the presence of AD- and BM-MSCs and their secretomes and a significant increase was observed when co-cultured with UC-MSCs and its secretome. Regarding the anti-inflammatory cytokine IL-9, HT-29 exhibited high expression levels, which decreased when co-cultured with various MSCs and their secretome, as illustrated in Fig. 6. In terms of the pro-inflammatory cytokines IL-17A and TNF-α, the levels of IL-17A decreased notably when co-cultured with AD- and UC-MSCs and their secretome. In contrast, co-

culture with BM-MSCs and its secretome significantly increased IL-17A levels (Fig. 6). TNF-α levels significantly increased upon treatment with BM-MSCs and UC-MSCs and their secretome but did not change when treated with AT-MSCs and its secretome.

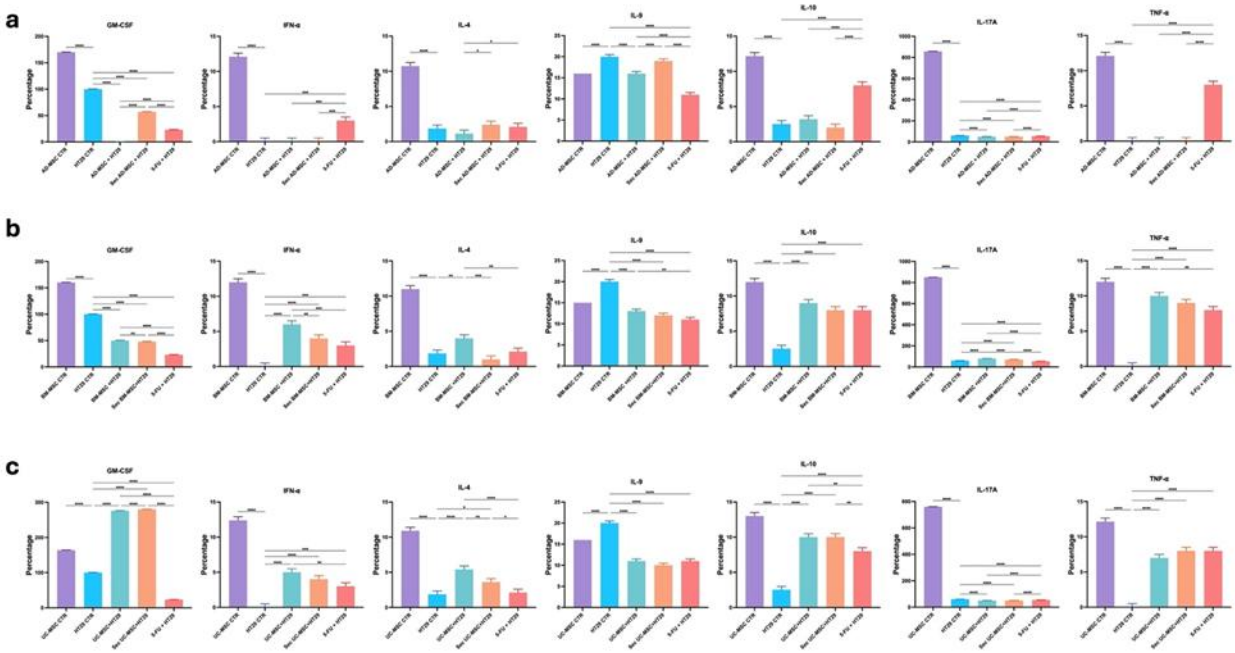

Figure 6: Anti- and pro- inflammatory cytokines expression in HT-29 cancer cells and upon co-culture with MSCs and their derived secretome. a-c Supernatants from the cell co-cultures were collected and undiluted samples were analyzed for cytokine detection, following the methods described. All data are shown as means ± SD, n=3, * p<0.05, ** p<0.005, ***p<0.001, ****p<0.0001.

Moreover, the expression of GM-CSF was found to be elevated in HT-29 colorectal cancer cells. However, when these cells were treated with AD- or BM-MSCs and their secretome, the levels of GM-CSF decreased significantly. In contrast, treatment with UC-MSCs and its secretome led to an increase in GM-CSF levels (Fig. 6). Additionally, treatment with BM-MSCs and UC-MSCs and their secretome led to an increase in the levels of IFN-α in HT-29 cells. However, there was no significant change in IFN-α levels when the cells were treated with AD-MSCs and its secretome.

Regarding HCT-116 cells, the levels of IL-4 remained unchanged between untreated cells and those co-cultured with MSCs and their secretome, except in the case of co-culturing with BM-MSCs' secretome, where a significant increase in IL-4 levels was observed. However, co-culture of HCT-116 cells with MSCs and their secretome resulted in increased IL-10 levels (Fig. 7). In terms of pro-inflammatory cytokines, IL-17A levels significantly decreased when HCT-116 cells were co-cultured with AD- and UC-MSCs and their secretome, but not with BM-MSCs and its secretome. TNF-α levels significantly increased upon treatment with BM-MSCs and UC-MSCs and their secretome but did not change when treated with AD-MSCs and its secretome. For the anti-inflammatory cytokine IL-9, HCT-116 exhibited high expression levels, which decreased when co-cultured with various MSCs and their secretome, as illustrated in Fig. 7. Conversely, the expression of GM-CSF was initially elevated in HCT-116 cells. However, treatment with various MSCs and their secretome led to a significant decrease in GM-CSF levels. Additionally, treatment with AD-MSCs and UC-MSCs and their secretome increased the levels of IFN-α in HCT-116 cells, whereas treatment with BM-MSCs and its secretome did not result in a significant change in IFN-α levels.

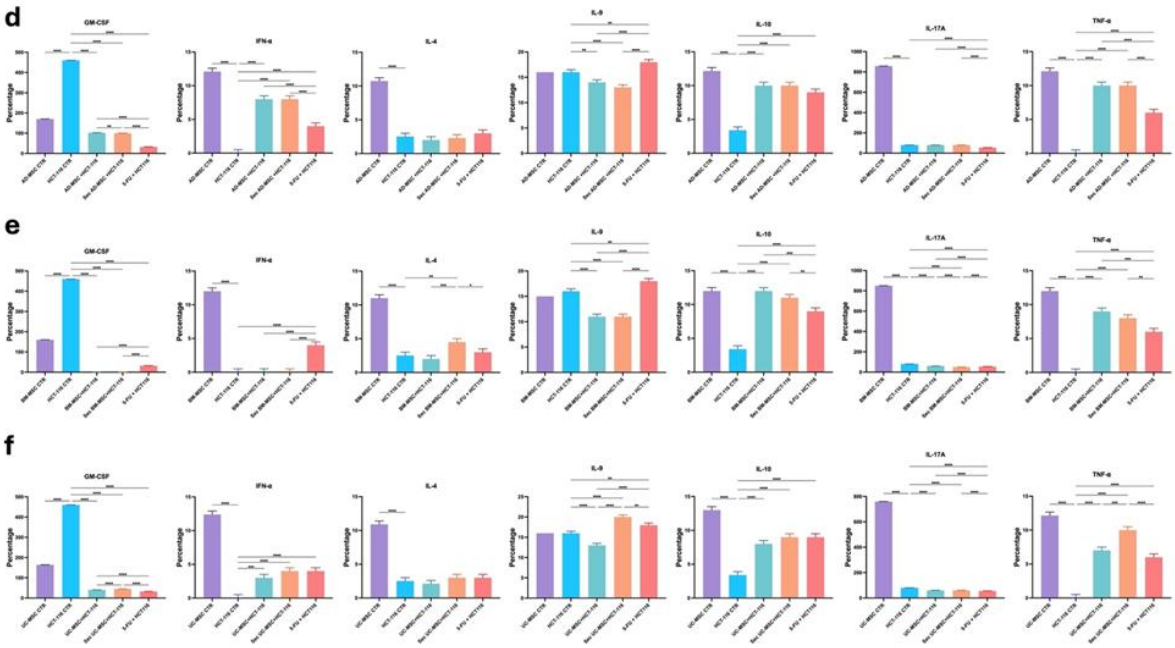

Figure 7: Anti- and pro- inflammatory cytokines expression in HCT-116 cancer cells and upon co-culture with MSCs and their derived secretome. d-f Supernatants from the cell co-cultures were collected and undiluted samples were analyzed for cytokine detection, following the methods described. All data are shown as means ± SD, n=3, * $p<0.05$, ** $p<0.005$, *** $p<0.001$, **** $p<0.0001$.

MTT Proliferation Test

To verify the cell line's authenticity, the cells were induced to differentiate into chondrocytes, adipocytes and osteocytes, as shown in the Fig. 8,9. Adipocytes were identified by the presence of Oil O Red, osteocytes were identified using Alizarin Red and chondrocytes were identified using Alcian Blue staining.

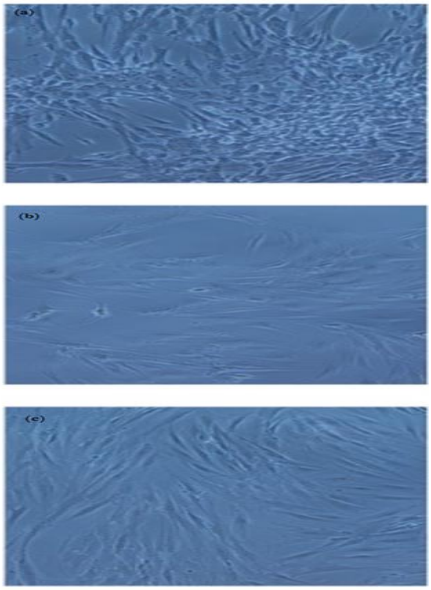

Figure 8: Differentiation capacity of MSCs. (a) Morphology of MSC from Umbilical Cord. Cells were incubated for 3 weeks with adipogenic, osteogenic and chondrogenic media. (b) Representative images of differentiated cells first row represent the day of coloration. The second row shows that the intracellular lipid droplets were confirmed by Oil Red O staining compared to the control cells. In the third row, the presence of calcium deposits was visualized by Alizarin Red staining compared to the control. The presence of GAG in the last row was confirmed with Alcian Blue staining and the solid chondrogenic micro mass compared to the control.

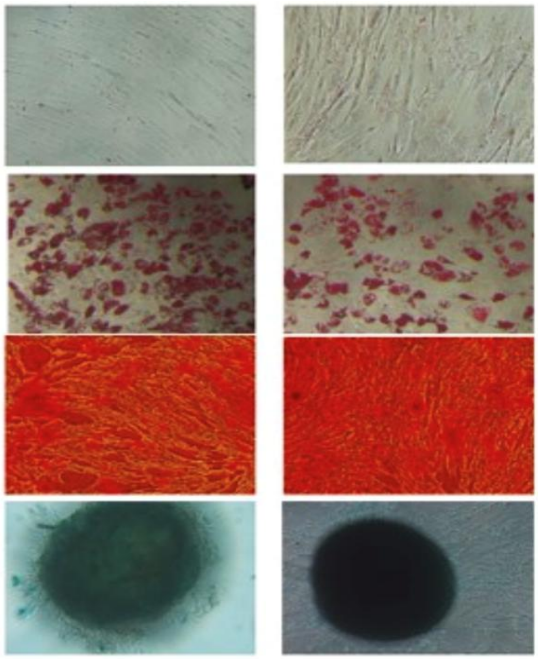

Figure 9: Characterization of MSC Differentiation capacity: morphological analysis and staining for adipogenesis, osteogenesis and chondrogenesis.

Cancer Cells Viability After Co-Culture with MSC or Their Derived Secretome

The cell viability analysis revealed a distinct response to various treatments, indicating that these conditions have different outcomes on the cells. When MSCs and both HCT-116 as well as HT-29 were co-cultured, we witnessed an evident effect upon cancer cell viability where it declined by 90% for HCT-116 and a proportionate drop of approximately 75% occurred for HT-29 compared to a control group without treatment. Upon subjecting these cells to the secretome derived from MSCs, we witnessed a 60% decrease in HCT-116 viability (as was noted upon exposure to actual MSCs) while a drop of approximately 90% occurred for HT-29. This noteworthy reduction in cell viability in both cancer cell lines indicates failure or breakdown within most cells causing cell death along with severe dysfunctions while being treated under such conditions. In terms of applying treatment with 5-FU however, it is evident that the observed effects were minimal in reducing the viability of HCT-116 cells whilst having no impact whatsoever on HT -29 cells. In conclusion, the utilization of 5-FU modestly affects cell viability; though when co-cultured with either MSC or secretome, the cell viability is impacted greatly (Fig. 10).

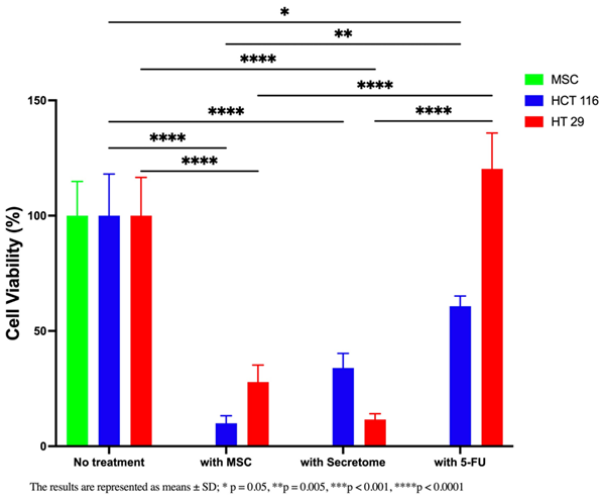

Figure 10: The effect of 5-FU, MSC and secretome on HCT116 and HT29 cell growth and viability. Cells were seeded in triplicates and treated with the indicated solutions. The MTT proliferation assay was performed and the results represent the average of 3 independent experiments.

Discussion

Colorectal Cancer (CRC) remains one of the most prevalent malignancies worldwide and continues to pose a major clinical challenge due to its high mortality rate. Tumor metastasis is a key determinant of CRC lethality and represents a significant obstacle in disease management [9]. In recent years, increasing attention has been directed toward the potential use of Mesenchymal Stem Cells (MSCs) and their secretome as therapeutic strategies to inhibit tumor growth and regulate metastasis. MSCs have emerged as promising candidates due to their immunomodulatory properties. Anti-inflammatory effects and differentiation potential [10]. Several studies have explored MSCs derived from Adipose Tissue (AT), Bone Marrow (BM) and Umbilical Cord (UC) as potential therapeutic tools in CRC [11].

In the present study, immunophenotyping and characterization of MSCs and CRC cell lines provided insight into their interactions within the Tumor Microenvironment (TME). All MSC populations expressed classical markers (CD44, CD73, CD105 and CD133) and lacked hematopoietic markers (CD45, CD34 and HLA-DR), consistent with established criteria for MSC identification. Notably, BM- and AT-MSCs exhibited higher expression CD105 compared to UC-MSCs, confirming the heterogeneity of MSC populations depending on their tissue of origin [12]. Comparative analysis of CRC cell lines (HT-29 and HCT-116) revealed distinct phenotypic differences. HT-29 exhibited elevated CD44 and CD133 expression compared to MSCs, reflecting their tumorigenic and stem-like properties, while CD105 expression was reduced in both cancer cell lines. Importantly, co-culture experiments demonstrated that MSCs and their secretomes significantly reduced CD44 expression in HCT-116 cells and decreased tumor markers such as CEA, AFP and CA-19-9 in HT-29 cells. These findings suggest a potential inhibitory eddect of MSCs on tumor proliferation and invasion, consistent with studies reporting MSC-mediated modulation of tumor phenotype [13].

Furthermore, MSCs and their secretome induced apoptosis and reduced cancer cell viability, with effects comparable to 5-Fluorouracil (5-FU), a standard chemotherapeutic agent for CRC. This highlights the potential of MSC-based approaches as alternative or complementary therapeutic strategies. However, previous studies have also demonstrated that MSCs can suppress anti-tumor immune responses, thereby facilitating tumor progression, emphasizing the dual role of MSC within the TME [14].

The analysis of CD3 expression provided additional insight into immune modulation. Although CD3 is typically expressed by tumor-infiltrating lymphocytes rather than cancer cells, both HCT-116 and HT-29 cell cultures initially exhibited detectable CD3 levels. Co-culture with MSCs, their secretome or treatment with 5-FU resulted in significant reduction in CD3 expression, suggesting modulation of T cell-related signaling within the TME. These findings underscore the complex interactions between MSCs, tumor cells and immune components.

Matrix remodeling is another critical aspect of CRC progression. Tissue Inhibitors of Metalloproteinase (TIMPs) regulate Matrix Metalloproteinase (MMPs), which are involved in extracellular matrix degradation and metastasis. In this study, MSCs upregulated TIMP expression in HT-29 cells, potentially contributing to reduced tumor invasion. While TIMP-1 and TIMP-2 are generally upregulated in CRC and associated with tumor progression, TIMP-3 is often downregulated and like to poor prognosis [15-18]. These findings suggest that MSCs may influence tumor progression through modulation of the MMP/TIMP balance. Cytokine profiling further highlighted the immunomodulatory role of MSCs in CRC. Cytokines such as IL-4, IL-10, IL-17A, GM-CSF, IFN-α and TNF-α are known to play critical roles in shaping the TME and influencing tumor progression [19].

Supportive Evidence

Our findings align with studies demonstrating the anti-inflammatory and immunomodulatory effect of MSCs. Co-culture with AT- and UC-MSCs and their secretomes resulted in decreased IL-17A levels, supporting their role in suppressing pro-inflammatory signaling with the TME [20-22].

Contradictory Evidence

In contrast, our results also revealed pro-inflammatory effects under certain conditions. BM-MSCs and their secretome significantly increased IL-17A and TNF-α levels, indicating a potential tumor-promoting role. This contradicts studies reporting consistent suppression of pro-inflammatory cytokines by MSCs and highlights their context-dependent behavior. Similarly, increased TNF-α levels following treatment with BM- and UC-MSCs diverge from reports of MSC-mediated inhibition of TNF-α signaling.

Explanation of Discrepancies

These conflicting findings may be attributed to differences in MSC source, tumor cell type and experimental conditions. The dual role of MSCs, exhibiting both pro- and anti-inflammatory effects, reflects their complex and context-dependent behavior within the TME.

Finally, transcription factors such as $FOXO_3$ have been implicated in CRC progression. While some studies report a tumor-suppressive role for $FOXO_3$, others suggest it promotes tumor invasion through regulation of matrix metalloproteinase [23,24]. This duality further illustrates the complexity of molecular pathways involved in CRC and highlights the need for further investigation.

Conclusion

In conclusion, this study provides important insights into the characterization and immunophenotypic profiles of Mesenchymal Stem Cells (MSCs), Colorectal Cancer (CRC) cell lines and their interactions within co-culture systems. The results demonstrate that MSCs, particularly depending on their tissue of origin, can modulate the Tumor Microenvironment (TME) through multiple mechanisms, including the regulation of tumor-associated markers, cytokine secretion profiles and the induction of apoptosis in cancer cells. Importantly, our findings highlight the dual and context-dependent role of MSCs, exhibiting both anti-tumor and pro-inflammatory effects, which underscores the complexity of their application in cancer therapy. While MSCs and their secretome show promising potential as therapeutic agents or adjuncts to conventional treatments such as 5-Fluorouracil (5-FU), their variable behavior necessitates careful consideration. Further studies are required to better understand the molecular mechanisms underlying MSC-mediated effects and to optimize their use in targeted and safe therapeutic strategies for colorectal cancer.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Statement

The Reviva Regenerative Medicine center Ethics Review Board approved the retrieval of all MSC collections (approval reference number: CTU-024-003) and all patients were asked to read and approve/sign informed consent forms prior to any participation.

Informed Consent Statement

All Authors approved the manuscript and give their consent for submission and publication.

Authors' Contributions

Conceptualization and supervision, C.K.; experiments and methodology, C.K.and J.G.; validation, C.K and F.H.; formal analysis, C.K and F.H.; investigation, C.K., J.G and F.H.; resources, C.K.; data curation, F.H and C.K..; writing—original draft preparation, C.K and F.H.; visualization, F.H and C.K.; Writing - Review and Editing, L.H., H.G., D.M., R.S., M.R., C.H., H.E.G., F.H and C.K.. All authors have read and agreed to the submitted version of the manuscript.

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