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Adipocytes Isolated from Visceral and Subcutaneous Depots of Donors Differing in BMI Crosstalk with Colon Cancer Cells and Modulate their Invasive Phenotype

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Abstract

PURPOSE: Mechanisms related the crosstalk between adipocytes and colon cancer cells are still not clear. We hypothesize that molecules and adipocytokines generated from the adipose tissue of obese individuals accentuate the effect on the metabolic reprogramming in colon cancer cells, i.e. induce disarray in energy metabolism networks of the targeted affected colonic epithelial cells, prompting their malignant phenotype. *METHODS:* To explore the mechanistic behind this crosstalk we conducted a co-culture model system using human colon cancer cells having different malignant abilities and adipocytes from different depots and subjects. *RESULTS:* The results demonstrate that co-culturing aggressive colon cancer cells such as HM-7 cells, with Visceral or Subcutaneous adipocytes (VA or SA respectively) from lean/obese subjects significantly up-regulate the secretion of the adipokines IL-8, MCP1, and IL-6 from the adipocytes. Surprisingly, the response of co-culturing HM-7 cells with obese SA was substantially more significant. In addition, these effects were significantly more pronounced when using HM-7 cells as compared to the less malignant HCT116 colon cancer cells. Moreover, the results showed that HM-7 cells, co-cultured with VA or SA from obese subjects, expressed higher levels of fatty acid binding protein 4; thus, the conditioned media obtained from the wells contained HM-7 cells and adipocytes from obese subjects was significantly more efficient in promoting invasion of HM-7 cells. *CONCLUSIONS:* We conclude that interaction between adipocytes and colon cancer cells, especially the highly malignant cells, results in metabolic alterations in colon cancer cells and in highly hypertrophy phenotype which characterized by increasing adipokines secretion from the adipocytes.

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Introduction

Obesity became an epidemic problem which can promote several health complications as cardiovascular disease, diabetes, steatosis and several kinds of cancers as colon cancer—the fourth cause of cancer death in the world [1,2].

The adipose tissue is an endocrine organ, which is functionally and metabolically heterogenic and can contribute to a variety of metabolic and medical conditions [1,3]. Responding to an external stimulation, the adipose tissue secretes cytokines known also as "adipokines". Adipokines regulate a wide array of metabolic and immune processes [4,5].

Obesity can promote an undergoing hypertrophy in the adipose tissue, which leads to the secretion of high amount of pro inflammatory adipokines as interleukin 6 (IL-6), interleukin 8 (IL-8), leptin, tumor necrosis factor α (TNF-α) and *monocyte chemoattractant protein-1 (MCP1)* [6]. Many of these adipokines are

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related to cellular pathways, which promote growth and survival of cancer cells as in colon cancer [7–10].

Two important fat depots of the adipose tissue are known, the visceral and the subcutaneous depots, which are morphologically and functionally different [11]. The visceral fat amount increases with age, it accounts for up to 5% to 20% of all body fat depending on gender, and it presents around abdominal viscera in mesentery and omentum. Subcutaneous fat accounts for 80% of all body fat and presents in subcutaneous areas [12].

Subcutaneous adipose tissue (SAT) was reported to be more sensitive to insulin, less lipolytic and more lipogenic than the visceral adipose tissue (VAT). On the other hand VAT has a higher level of lipid turnover, lesser amount of preadipocytes with differentiation capacity; it is infiltrated with cells of the immune system such as T-lymphocytes and macrophages, and of inflammatory cytokines, thus VAT is more vascular than SAT [11,13–15].

The fact that visceral fat is in close vicinity to important organs in the body such as the colon, the liver and the heart, can explain the important role of this depot in promoting pathophysiological alterations such as metabolic syndrome and cancer [1,16]. The direct drainage of free fatty acids and adipokines from the visceral tissue direct to the liver can activate immune responses leading to secretion of inflammatory mediators [12]. Moreover, these mediators along with pro inflammatory adipokines can constituently stimulate several mutagenic pathways in close tissues as colon and mediate growth, proliferation and invasion of the cells [17].

Taken together, the facts presented herein suggest that there is an important network between the adipose tissue, especially from VAT depots in obesity, and the colonic tissue surrounding it.

In this study, using a co-culture system model, we showed the mutual interactions between human adipocytes (VA/SA, lean/obese) and colon cancer cells (of different malignancy levels), and suggest a putative molecular mechanism of the nature of this interaction. We showed that adipocyte-primed cancer cells promote the adipocytes to secrete higher level of pro inflammatory adipokines, which ultimately contribute to a more malignant phenotype of the cancer cells.

We compared the levels of different adipokines in the conditioned media (CM) collected from the co-culture system. We demonstrate that CM collected from the co-culture system, promotes invasiveness of colon cancer cells. Interestingly, these effects depend on the body mass index (BMI), adipocytes type (SA, VA), and the adipokine released.

As far as we know this is one of a few studies to use a unique system of co-culturing human adipocytes, from different depots of different BMI, with colon cancer cells of various malignancy levels. This novel approach allows to better explore the effect of adipocyte microenvironment and close interactions between these cell types, and to help in exploring new therapeutic strategies.

Materials and Methods

Materials

All chemicals and biochemicals were from Sigma Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Cell Culture

The human colon cancer cell lines HCT116 and HM-7 were cultured at 37 °C, 5% CO_2 in DMEM supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 0.2% (v/v) penicillin streptomycin. HCT116 cells were obtained from the American Type Culture Collection (ATCC, USA). HM-7 is a cell variant of LS174T, previously selected for its capacity to produce high amounts of mucin and to be highly metastatic in *in vivo* [18] and *in vitro* systems [19].

Isolated human preadipocytes received as a generous gift from Zen-Bio (Research Triangle Park, NC, USA). The cells provided were isolated by Zen-Bio from human subcutaneous or visceral adipose tissue, from lean or obese subjects (for details see Table 1). Zen-Bio is committed to provide highest quality isolated primary human cultured preadipocytes and adipocytes. The cells were treated and differentiated according to the manufacturers' instructions. Briefly, adipocytes are differentiated *in vitro* from preadipocytes using Zen-Bio's patent protected differentiation methods after culturing on six wells plates. Completely confluent wells were treated with the differentiation media provided by Zen-Bio for 7 days and then the media are replaced by adipocyte's media provided also by Zen-Bio and changed every alternate day. Adipocyte differentiation was achieved by day 14 of treatment with the differentiation media, according to microscopic features (See Figure 1, A–D) and expression of Fatty Acid Binding Protein 4 [FABP4], see Figure 1*E*). FABP4 also known as aP2 is the predominant FABP isoform found in adipocytes and is often used as a marker for adipocyte differentiation.

Co-Culture Assays

Tumor cells and adipocytes were co-cultured using a Transwell culture system (0.4-μm pore size; BD Bioscience). 4×10^5 (HCT116), or 4.2×10^5 (HM-7) cells were seeded in the top chamber of the Transwell system in DMEM for 24 hours then media were changed to adipocytes serum-free medium containing 0.1% (w/v) of delipidated bovine serum albumin (BSA) in the presence or absence of human mature adipocytes from different subjects with different body mass index (Lean/Obese) and from a different fat depot (Subcutaneous/Visceral) which were cultured in the lower chamber. Incubation of the cells in the Transwell system allowed the diffusion of soluble factors. Adipocytes or cancer cells were separately cultured in similar conditions served as controls. After 24 hours conditioned media (CM) were collected from: adipocytes previously co-cultivated during 24 hours with cancer cells, mature adipocytes or cancer cells which were separately cultured, filtered and stored at −80 C° for further use (Supplementary Figure 1).

The collected CM (CM-L, CM-F, CM-L + HM-7/HCT116, CM-F + HM-7/HCT116) was used to assess its effect on transcriptional levels of several adipokines and on cellular bioenergetics and invasive properties of colon cancer cells. Both colon cancer cells and adipocytes separately cultured or cultured in the co-culture system were lysed by trizol reagent (Tri Reagent) and used for gene and protein isolation. Protein concentration was determined by Bradford assay [20].

Table 1. Cell type and donors characteristics

Cell name	Tissue type	Gender of donor	Age	BMI	Medicines
SPF1	Adipose -Subcutaneous	Female	40	23.3	UN
SPF3	Adipose -Subcutaneous	Female	33	35	NA
OPF1	Adipose- Omental/visceral	Female	28	26.6	Synthroid
OPF3	Adipose Omental/visceral	Female	38	40.2	Vitamin D
HCT116	Colon—cancer cells	Male	adult	NA	NA
HM7	Colon—aggressive cancer cells	Female	58	NA	NA

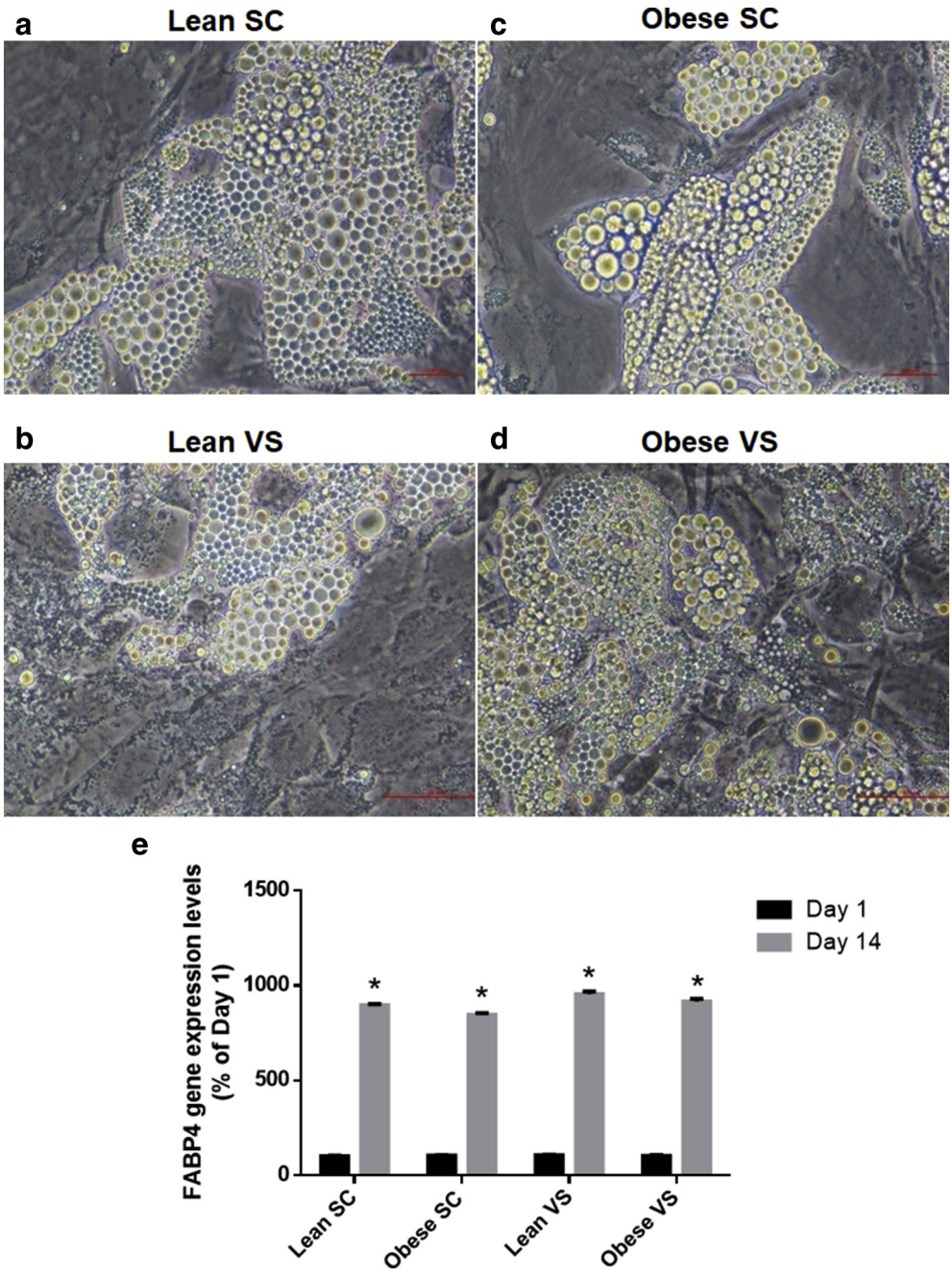


Figure 1. Differentiated human adipocytes. The cells were treated with the differentiation media provided by Zen-Bio. The photomicrographs show representative cells on day 14 of each treatment. (a), differentiated adipocytes from SC adipose tissue of a lean subject. (b), differentiated adipocytes from VS adipose tissue of a lean subject. (c), differentiated adipocytes from SC adipose tissue of an obese subject. (d), differentiated adipocytes from VS adipose tissue of an obese subject. Scale bar: 50 μm**.** Adipocytes at day 1 and 14 days after culturing in differentiation media were harvested and tested for the adipogenic transcriptional factor FABP4 using quantitative real-time PCR. Figure 1E shows the results of the expression of FABP4 on day 14 as compared to day 1 of differentiation. *$P < .00001$ (Student's *t* test).

HCT116 and HM-7 results were presented independently and were compared to its controls.

ELISA

All adipokines levels in the CM were determined using LXSAHM-05 Human Magnetic Luminex Screening Assay (5 PLEX) (R&D Systems, Minneapolis, USA) according to the manufacturers' instructions.

Boyden Chamber Invasion Assay. Cells invasion was measured as previously described [21] with some modifications. Briefly, HM-7 cells suspension (450 μl, 2.5×10^5 cells) was seeded on the top of Matrigel-coated (invasion assay) 8-μm pore diameter Transwell plate from BD Bioscience. The CM from differentiated adipocytes was

placed in the lower chamber. For these studies we exposed the HM-7 cells to 100% CM (0:100; DMEM:adipo CM) to promote significant invasion. Twenty-two hours later, cells invading the lower chamber were stained with 0.1% (w/v) crystal violet (60% PBS, 40% ethyl alcohol) and counted with an inverted microscope. The results from one independent experiment in duplicate are presented.

Cell Respiration Measurements. Cellular oxygen consumption rate (OCR) was measured using the XF24 Analyzer Seahorse Bioscience, Massachusetts, USA) as described previously [22]. For maximal OCR, 0.4 μM carbonyl cyanide-4-trifluoromethoxy-phenylhydrazone (FCCP) was used. Optimal FCCP concentration was determined in preliminary experiments. For these studies we exposed the HM-7 cells to 50% CM (50:50; DMEM:adipocytes CM).

RNA Extraction and Real-Time PCR. RNA was isolated using Tri Reagent solution (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription was performed using a High-Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA) with random primers on a Veriti® 96-well Thermal Cycler (Applied Biosystems). Real-time PCR was performed using SYBR® Green (Applied Biosystems) in an ABI PRISM® 7300.

Primers are listed in Supplementary Table 1. All results were normalized to the expression of the *GAPDH* gene.

Data Analysis

Statistical analyses were performed by one-way ANOVA Dunnett's test and by two-tailed Student's *t* test. Statistical analyses were carried out using GraphPad Prism software and the *P* values indicated. Results are presented as mean ± SEM. All figures show representative results of at least two independent experiments. Differences were considered significant if $P < .05$. Each adipocyte cell line was obtained from one subject.

Results

Human Preadipocytes Differentiation In Vitro

Human isolated preadipocytes (Zen-Bio, Research Triangle Park, NC, USA) from subcutaneous (SC) or visceral (VS) adipose tissue, from lean or obese subjects were cultured on six-well plates. The cells were treated and induced to differentiate according to the manufacturers' instructions. Twenty-four hours later, when the wells were completely confluent, the cells were treated with a differentiation media provided by the manufacturer. Seven days later, the media was replaced by adipocytes media and changed every other day. Adipocytes differentiation was observed by day 14. Figure 1, *A* and *B* exemplifies SC and VS adipocytes cultures from lean donors after terminal differentiation; Figure 1 *C* and *D* exemplifies SC and VS adipocytes cultures from obese donors after terminal differentiation. Scale bar = 50 μm. Differentiation stage of the adipocytes was confirmed by extent of FABP4 expression on day 14 of culture as compared to day 1, see Figure 1*E*.

Co-Culturing of Colon Cancer Cells with Mature Adipocytes Stimulates them to Secrete More Adipokines

We first tested whether the addition of colon cancer cells to cultured adipocytes would affect the levels of secreted adipokines by the adipocytes [23,24].

Using the co-culture cell system described in Materials and Methods, we cultured preadipocytes from different depots (VS or SC) and from different subjects (lean (L) or obese (F)) on the bottom chamber. The preadipocyte culture was treated with differentiation media (see above) until the culture reached full differentiation (See Figure 1), and only after reaching this point, we added colon cancer cells (HM-7 or HCT116) on the top chamber for 24 h. Twenty-four hours prior to CM collection, cells were cultured with serum-free medium to allow the diffusion of soluble factors between the chambers. Then, CM was collected and the levels of TNF-α, leptin, MCP1, IL-8 and IL-6 were measured. The results are summarized in Figure 2, *A*−*D*.

A higher level of IL-8, IL-6 and MCP1 adipokines were detected in the CM where HM-7 cancer cells were present in the co-culture as compared to the levels detected in CM where HCT116 cancer cells were present in the co-culture. Actually, it seems like the presence of HCT116 cells did not affect these adipokines secretions to the CM (Figure 2, *A*−*C*).

Interestingly, when HM-7 cells and visceral-derived adipocytes from lean or obese subjects were co-cultured (Figure 2, *A*−*D*), a 7.7-fold-increase of IL-8 levels in L + HM-7-CM and 2-fold-increases in IL-8 levels in F + HM-7-CM were detected compared with the levels secreted in CM collected from cultures of lean or obese adipocytes, respectively (Figure 2*C*). Surprisingly, the presence of adipocytes from lean subject with cancer cells had a stronger and more significant effect on the adipokines levels compared to the levels secreted in CM collected from co-cultured obese adipocytes and cancer cells. When HCT116 cells were co-cultured with adipocytes from lean or obese subjects (Figure 2, *A*−*D*), the levels of IL-6 and IL-8 adipokines did not reach even detectable levels (Figure 2, *B* and *C*).

When we compared the increase in secretion of the different adipokines in the presence of colon cancer cells, leptin levels showed the smallest changes (Figure 2*D*) and TNF-α was not detected in any of the collected CM (data not shown).

A similar trend was detected when cancer cells were co-cultured with subcutaneous-derived adipocytes (Figure 2, *A*−*D*). Similar effects as for MCP1 (Figure 2*A*) levels were measured for IL-6 (Figure 2*B*) and IL-8 levels (Figure 2*C*). Still the presence of adipocytes from obese subject with cancer cells had a stronger and more significant effect on the adipokines levels compared to the levels secreted in CM collected from co-cultured lean adipocytes and cancer cells. The largest effect was detected when HM-7 colon cancer cells where co-cultured with adipocytes isolated from an obese subject, reaching a 32-fold-increase in IL-8 levels (Figure 2*C*).

Co-culture of SC adipocytes from lean (L) subject with HCT116 cells resulted in the highest levels of leptin (Figure 2*D*). The levels of leptin were consistently higher in CM acquired from SC adipocytes than VS adipocytes of either obese (F) or lean (L) subjects. Overall, these results demonstrate that there is a very strong interaction between adipocytes and colon cancer cells, especially between the highly malignant cells (HM-7) and the adipocytes, and the result of this interaction is the secretion of higher concentrations of specific adipokines as MCP-1, IL-8 and IL-6.

We additionally measured mRNA expression levels of the adipokines IL-8 and MCP1 in adipocytes from lean or obese subjects co-cultured with HM-7 cells. Figure 3 showed an increase of IL-8 and MCP1 mRNAs expression in adipocytes from lean or obese subjects co-cultured with HM7 cells (L + HM-7 and F+ HM-7). The levels were significantly higher than the levels expressed by separately cultured adipocytes from lean or obese subjects (L or F) (Figure 3, *A*−*H*).

When we used VA, the expression levels of IL-8 were up-regulated by 50-fold in L + HM-7 and F + HM-7 (Figure 3 *A* and *B*) and were

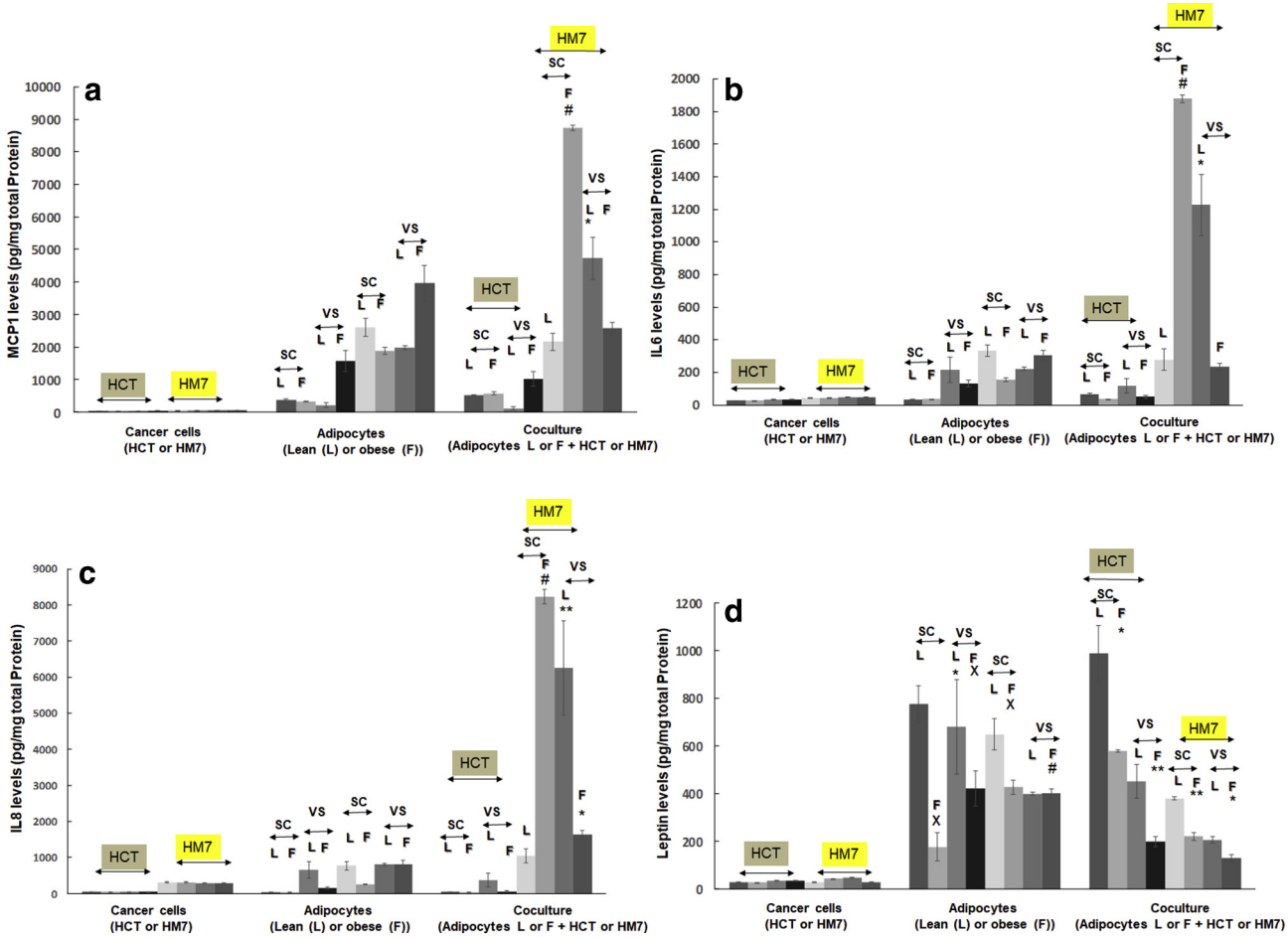


Figure 2. Co-culture of colon cancer cells with mature adipocytes stimulates them to secrete more adipokines. Cancer cells (HM-7/HCT116), and visceral adipocytes (VA) or subcutaneous (SA) adipocytes from lean (L) or obese (F) subjects were co-cultured on Transwells or were separately cultured in 6 wells for 24 h. Conditioned media (CM) was collected and several adipokines were measured. (a) MCP1 (b) IL6 (c) IL8 (d) Leptin levels were presented as pg/mg total protein in cells.(a,b), $^{\#}P < .001$ (Student's *t* test) compared to co-cultured lean adipocytes and separately cultured F adipocytes, $^{*}P < .05$, compared to co-cultured obese adipocytes and separately cultured L adipocytes. (c), $^{\#}P < .001$ (Student's *t* test) compared to co-cultured lean adipocytes and separately cultured F adipocytes, $^{*}P < .05$, compared to co-cultured lean adipocytes and separately cultured F adipocytes. $^{**}P < .05$ (Student's *t* test) compared to separately cultured L adipocytes.(d), $^{x}P < .05$, $^{\#}P < .001$ (Student's *t* test) compared to separately cultured L adipocytes, $^{*}P < .05$, $^{**}P < .01$ (Student's *t* test) compared to co-cultured lean adipocytes.

up-regulated by 5 and 7-fold of MCP1 expression levels respectively (Figure 3, *C* and *D*) as compared to separately cultured adipocytes (L or F).

When we used SA, the same trend was observed for IL-8 and MCP1 expression levels. These adipocytokines were up-regulated by 150 and 20-fold in F + HM-7 cells respectively as compared to separately cultured obese adipocytes (F) (Figure 3, *F* and *H*). However, the fold changes extent in the adipokines levels were lower when adipocytes from lean subjects were used since the expression levels of the adipokines IL-8 and MCP1 were up-regulated only 12 and 6 folds respectively in L + HM-7 cells as compared to separately cultured lean adipocytes (L) (Figure 3, *E* and *G*). The mRNA adipokines levels in adipocytes or cancer cells separately cultured were constitutively lower (Figure 3, *A*–*H*). This indicates that the co-culture is an important stimulator of adipokine transcription. It is noteworthy that the transcript expression level of MCP-1 and IL-8 in cancer cells cultured in media with no additions was negligible (data not shown).

The overall results indicate that co-culturing HM-7 cells with adipocytes up-regulate the transcription level of the adipokines IL-8 and MCP1. This can explain the higher levels of the secreted adipokines detected in the CM (Figure 2).

CM from VA and SA of Obese Subjects Increases the Invasiveness Ability of HM-7 Cells

Since adipokines are known to promote invasion and migration of cancer cells, we evaluated the ability of CM collected from adipocytes isolated from obese and lean subjects to promote invasion of HM-7 cells. We therefore collected CM from transwells of SA or VA from obese subjects, which underwent co-culture with HM-7 cells (CM-F + HM-7). Figure 4 shows that CM-F + HM-7- stimulated the invasive properties of HM-7 cells by 2.5 and 1.5-fold, respectively (Figure 4, *A* and *B*). No significant change was seen when treating HM-7 cells with CM from transwells of adipocytes derived either from SA or VA from lean subjects following co-culture with HM-7 cells (CM-L + HM-7) (Figure 4, *C* and *D*). However, the invasive

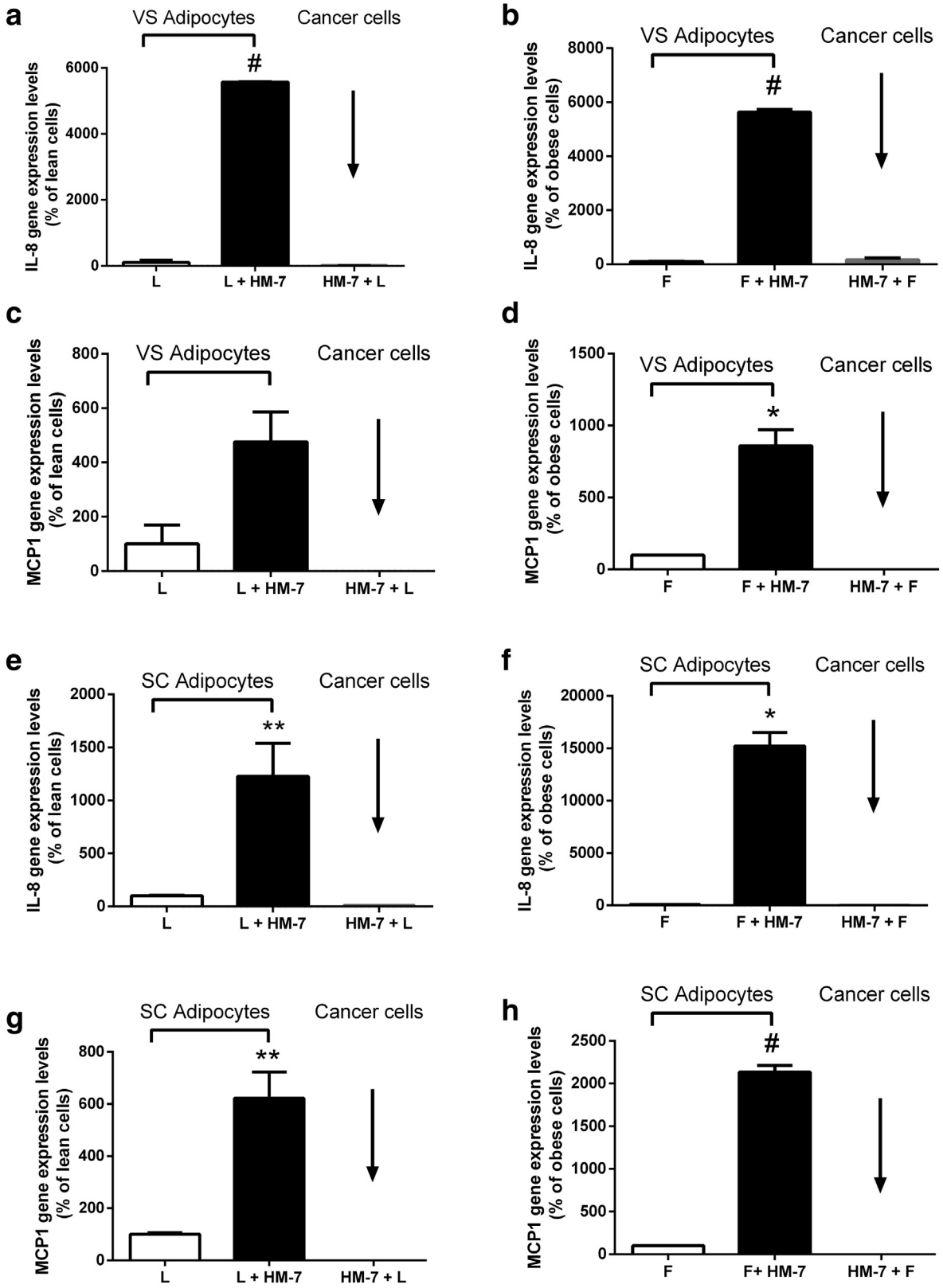


Figure 3. Cancer cells induce higher expression of adipokines from the different adipocytes. Gene expression was detected using quantitative real-time PCR in visceral adipocytes (a-d) or in subcutaneous adipocytes (e-h), from lean or obese subjects co-cultured with HM-7 cells. L + HM-7 represent gene expression in adipocytes from lean subject co-cultured with HM-7 cells.F + HM-7 represent gene expression in adipocytes from obese subject co-cultured with HM-7 cells. HM-7+ L (cancer cells) represent gene expression in HM-7 cells co-cultured with adipocytes from lean subject. HM-7+ F (cancer cells) represent gene expression in HM-7 cells co-cultured with adipocytes from obese subject. The results are presented as % of control (Lean (L) /obese (F)). $^{**}P < .05$, $^{*}P < .01$, $^{\#}P < .001$ (One way ANOVA- Dunnett's test).

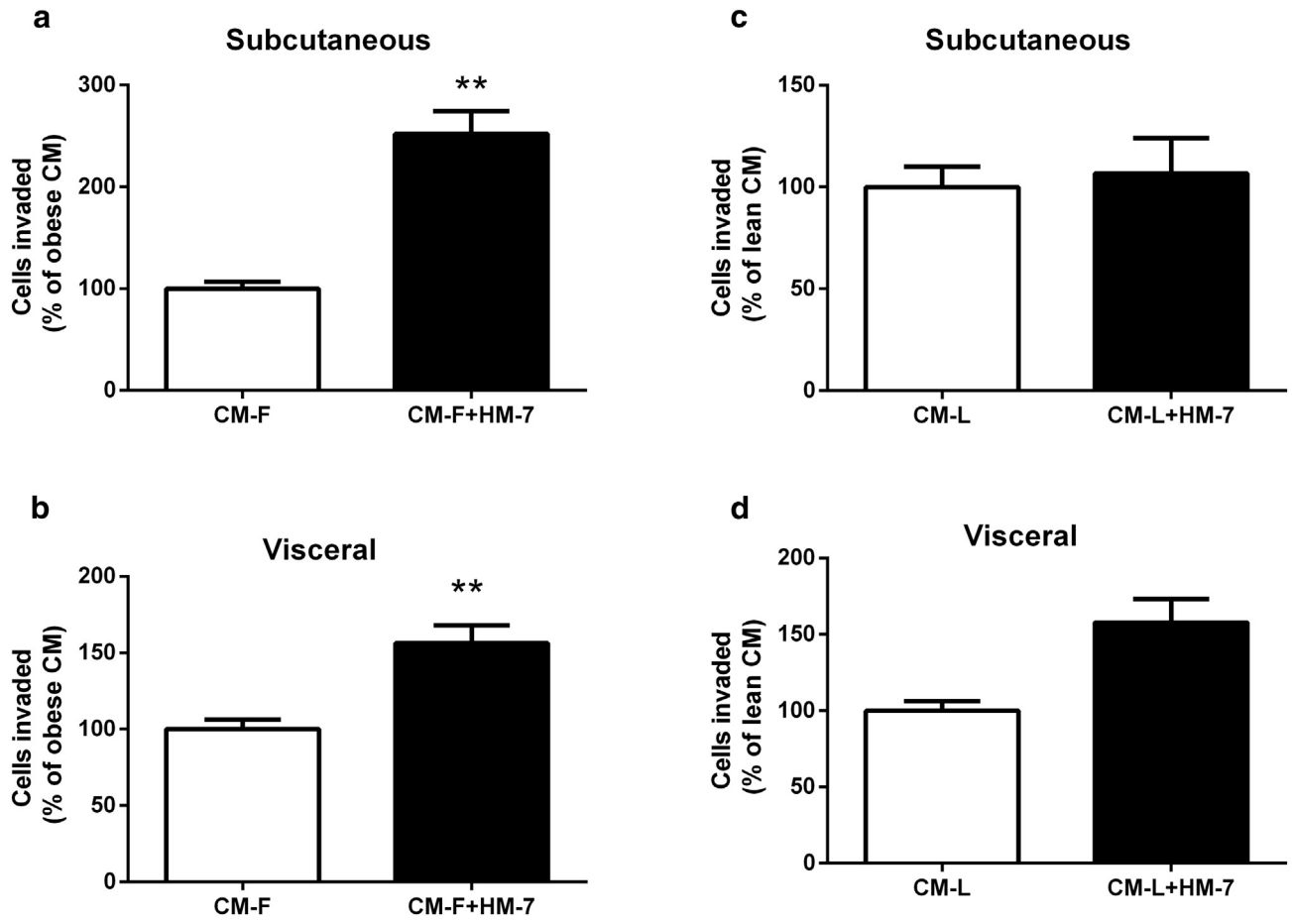


Figure 4. CM from VA and SA of obese subjects increases the invasiveness ability of HM-7 cells. HM-7 cells (450 µl, 2.5×10^5 cells) were seeded on the top of Matrigel-coated transwell plate and CM from subcutaneous (a, c) or visceral (b, d) differentiated adipocytes were placed in the lower chamber. CM-F or CM-L represent HM-7 cells treated with conditioned media collected from wells containing only adipocytes from obese /lean subjects. The comparison was performed between this control and the effect of CM collected from co-cultured wells of adipocytes and HM-7 cancer cells (CM-F + HM7 or CM-L + HM-7). Invasion of HM-7 cells towards CM was assessed. Bars report mean fold change ± SEM. Results are presented as % of control. One of three experiments are presented. $^{**}P < .05$ vs. control (CM-L or F) (Student's *t* test).

ability of HM-7 cells was significantly more pronounced when they were treated with CM from transwells of VA from obese subjects following separately cultured or co-culture with HM-7 cells as compared to CM from transwells of SA from obese subjects separately cultured or co-culture with HM-7 cells. Collectively, visceral CM was significantly more efficient in promoting invasion of colon cancer cells than subcutaneous CM (see also Table 2).

Table 2. Assessment of number of invading colon cancer cells

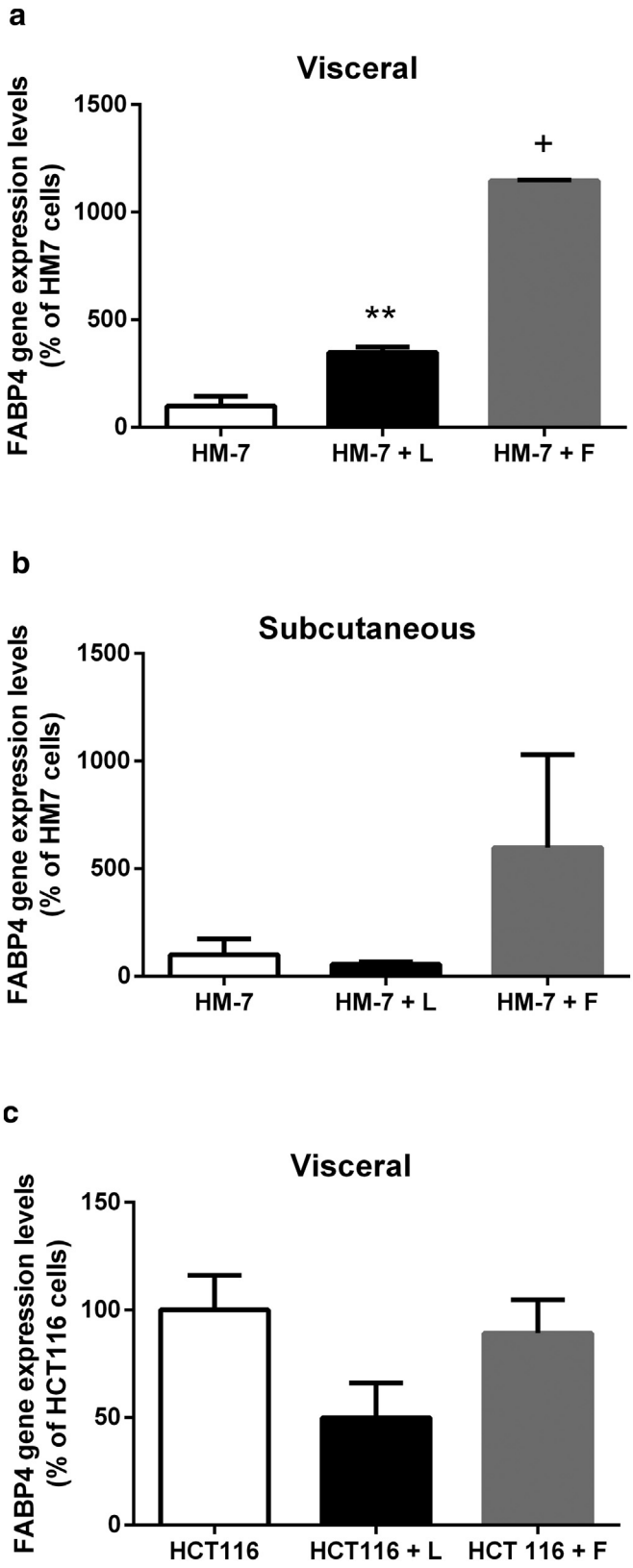
	CM-F	CM-F + HM-7
SC	83 ± 5.5	$210.5 \pm 18.5^{\#}$
VS	$302 \pm 19^{**}$	$472 \pm 35^{*}$

A suspension of HM-7 cells (2.5×10^5 cells in 450 µl growth media) were seeded on the top of a Matrigel-coated transwell plate and CM from differentiated adipocytes was placed in the lower chamber. Invasion of HM-7 cells towards adipocyte-conditioned medium (CM) were assessed. SC: subcutaneous adipocytes, VS: visceral adipocytes, CM-F: conditioned media collected from wells containing adipocytes of an obese subject. CM-F + HM-7: conditioned media collected from Transwells containing HM-7 cells and adipocytes from an obese subject. Statistical analyses were performed by two-tailed Student's *t* test. $^{**}P < .01$ as compared to CM-F of SC, $^{*}P < .05$ as compared to CM-F + HM-7 of SC, $^{\#}P < .001$ as compared to CM-F of SC.

Visceral Adipocytes Increase the Transcription Level of Fatty Acid Binding Protein 4 in HM-7 Cells

Fatty acid binding protein 4 (FABP4) is a fatty acid-binding protein that recently was found to be up-regulated in ovarian cancer cells at the adipocyte-tumor cell interface, [24] suggesting its role as a key protein in omental-mediated metastatic ability, which may regulate metastasis and lipid metabolism in adipocyte—colon cancer cell interface. To this end, we tested the expression of FABP4 levels in HM-7 cells co-cultured with different adipocytes. Additionally, we tested the expression levels of genes that correlate to lipid metabolism, Acyl-CoA Oxidase 1 (ACOX1) and Carnitine palmitoyltransferase I (CPT1) to test whether cancer cells which are in adipocyte—colon cancer cell interface, up-regulate their lipid metabolism by oxidizing fatty acids acquired from adipocytes in purpose to grow and proliferate. We therefore tested the expression levels of the gene Hormone sensitive lipase (HSL) to assess whether the adipocytes in adipocyte—colon cancer cell interface up-regulate the lipolytic process, providing high levels of fatty acids to the cancer cells, as shown in previous study [24].

Figure 5*A* demonstrates that the levels of FABP4 were significantly up-regulated when HM-7 cells were co-cultured with VA from lean (HM-7 + L, 4-fold) or obese (HM-7 + F, 11-fold) subjects, as compared to the levels recorded in separately cultured HM-7 cells (Figure 5*A*). The same trend was detected in HM-7 cells co-cultured with SA, but it did not reach statistical significance (Figure 5*B*).



On the other hand, no increase in the FABP4 was detected in HCT116 cells co-cultured with adipocytes from VA either from obese (F) or lean (L) subjects, similarly to HCT116 cells co-cultured with adipocytes from SA from obese or lean subjects (not shown) indicating that in less aggressive cells VS-derived adipocytes exert less dramatic neoplastic-associated effects. No significant effect was measured for the expression levels of ACOX1 and CPT1 (Supplementary Figure 2, *A*–*D*).

Interestingly, we did not see any significant increase in the expression levels of HSL gene in the different adipocytes co-cultured with HM-7 cells (Supplementary Figure 3, *A*–*D*).

The unchanged trends in the lipid metabolism genes which we tested here can indicate that neither the cancer cells nor the adipocytes in the adipocyte—colon cancer cell interface do not up-regulate their lipid metabolism, indicating that there are additional mechanisms used by the cancer cells that promote their growth, survival and malignant abilities.

CM Harvested from the Co-Culture System Down-Regulates the Mitochondrial Function of HCT116 Cells

We next aimed at measuring whether the mitochondrial function of colon cancer cells exposed to different CM's is altered. We based this assumption on the results that the CM harvested from co-cultures modify the expression of genes involved in metabolism together with our previous findings showing that CM collected from adipose tissue of obese human or mice, induce a significant decrease in the mitochondrial function of colon cancer cells [16,25]. In this study, our objective was to test whether CM collected from the co-culture system in transwells of adipocytes and cancer cells, containing high concentrations of different adipokines, would affect the mitochondrial respiration of colon cancer cells. The effect was tested by assessing the oxygen consumption rate (OCR) of the colon cancer cells.

We demonstrate herein that incubation of HCT116 with CM collected from transwells containing adipocytes from obese VA and HCT116 cells (CM-F + HCT116), resulted in a significant decrease of 40% in OCR (Figure 6*A*). No change was detected in maximal OCR, measured following the addition of the strong mitochondrial uncoupler FCCP (Figure 6*B*). Similarly, a decrease of 40% in OCR was detected in HCT116, treated with CM collected from transwells obtained from co-cultures of adipocytes from obese SA and HCT116 cells (CM-F + HCT116), with no change in maximal OCR (Figure 6, *C* and *D*).

CM collected from transwells containing adipocytes from VA of an obese subject and HM-7 cells showed no change in OCR and the maximal OCR as compared to control (Figure 6, *E* and *F*). However, CM collected from transwells containing adipocytes from SA of a lean subject and HM-7 cells showed significant increase in OCR as

Figure 5. FABP4 over expressed in HM-7 cells co-cultured with visceral adipocytes cellsHM-7 (a, b) or HCT116 (c), cells co-cultured with visceral adipocytes (a,c) or subcutaneous adipocytes (b) from lean (HM-7/HCT116 + L) or obese subjects (HM-7/HCT116 + F). Twenty-four hours later cells were lysed and gene expression level was detected using quantitative real-time PCR. HM-7/HCT116 cells indicates control- cells which separately cultured, n = 3 wells from each treatment. Results are presented as % of control (HM-7/HCT116). $^{**}P < .05$, $^{+}P < .0001$ (One way ANOVA-Dunnett's test).

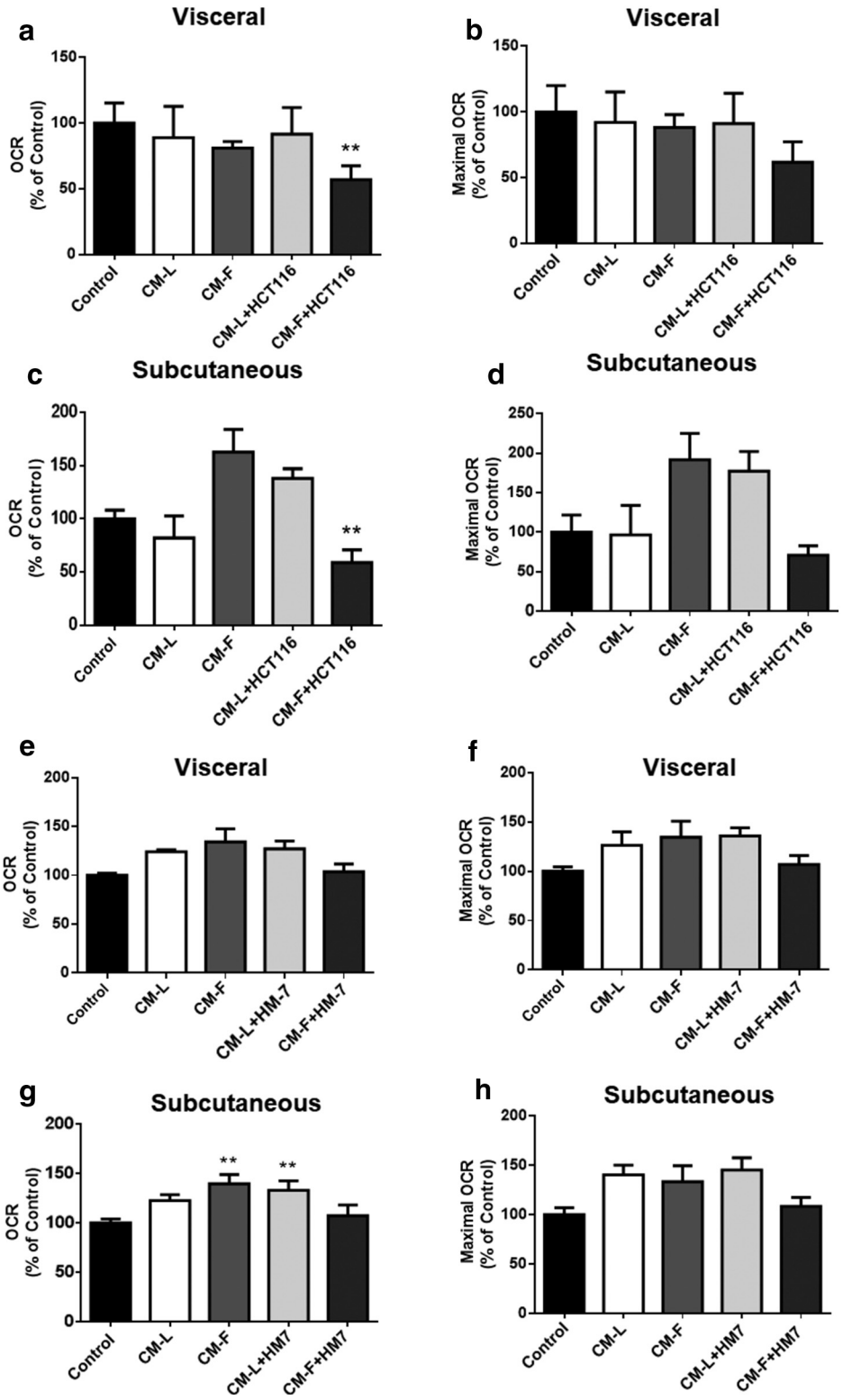


Figure 6. CM harvested from the co-culture system down-regulates the mitochondrial function of HCT116 cells(a-d), HCT116 (4×10^4) and (e-h), HM-7 cells (4.2×10^4) were treated for 24 hours with CM which were collected from: wells contained visceral adipocytes (a,b and e,f) or subcutaneous adipocytes (c,d and g,h) from obese and lean subjects and HCT116 or HM-7 colon cancer cells (CM-F/CM-L + HCT116, CM-F/CM-L + HM-7) or with CM which were collected from wells contained visceral adipocytes or subcutaneous adipocytes from obese (CM-F) or lean (CM-L) subjects which separately cultured.Cells were analyzed for their oxygen consumption rate (OCR) and maximal OCR following administration of 0.4 μM FCCP using the XF24 Analyzer. Cells which treated with regular adipocytes medium were used as control. n = 4 of each treatment. $^{**}P < .05$ vs. control (One-way ANOVE-Dunnett's test). Results are presented as % of control. Results of two independent experiments are shown.

compared to controls. The same trend was seen when treating the cells with CM-F with no significant change in maximal OCR in any of the CM treatments. These results are indicative of a more robust β-oxidation process (Figure 6*G*).

Discussion

The adipose tissue releases high levels of adipokines, lipids and fatty acids providing a very rich microenvironment around the tumor that can promote the cancer cells to grow and propagate [26]. Several adipokines related to low chronic inflammation conditions are over expressed in obesity and found in adipocytes- cancer cells microenvironment [27]. Therefore, the adipocytes are recognized as important regulators of tumor growth.

Several studies tested the crosstalk between cancer cells and adipocytes [24,27,28]. Dirat et al (2011) tested the crosstalk between breast cancer cells and human or murine adipocytes and found that adipocytes co-cultured with cancer cells modified the breast cancer cells characteristic leading these cells to a more aggressive behavior [27]. Nieman et al (2011), tested the crosstalk between human visceral adipocytes from different subjects and ovarian cancer and found that FABP4 play a key role in the metastatic behavior of this kind of cancer and that adipocytes surrounding cancer cells provide fatty acids to the cancer cells to up-regulate their rapid growth ability [24].

In this study, we tested the crosstalk between human adipocytes and colon cancer cells using a unique co-culture system to simulate cellular environment in the body. We tested the behavior of different adipocytes, considering body mass index of the donor-subjects and the type of the adipose tissue (visceral vs. subcutaneous), from which the adipocytes were isolated. We also used two colon cancer cell lines (HCT116 vs. HM-7), differing in their malignant ability.

Our results demonstrate that when co-culturing adipocytes with colon cancer cells, a significantly higher level of the adipokines IL-6, IL-8 and MCP1 are detected in the collected conditioned media (CM), with no significant change in leptin level. TNF-α level were undetectable in the collected CM.

Nieman et al (2011), showed that IL-6, IL-8 and MCP-1 were three of five cytokines most abundantly secreted by omental adipocytes. Based on these data we tested these adipokines in our co-culture system [24].

Our results are in line with several studies [27,29]. Dirat et al (2011) used human breast cancer cells and murine adipocytes and showed that the levels of IL-6 and IL-1β are significantly higher in the supernatant of co-cultured cells as compared to control. Similarly to our findings, the levels of TNF-α were undetectable in both co-cultivated and control cells [27]. Picon-Ruiz et al (2016) used mature human adipocytes and breast cancer cells and demonstrated elevated levels of IL-8, IL-6 and MCP1 in the CM of the co-cultured cells similarly to our results [29].

We report herein that the highest genes levels detected in the adipocytes corresponded to the highest levels of adipokines detected in the CM of the respective adipocytes. The results related to the expression levels of genes which were detected only in adipocytes and not colon cells are indicative that indeed, adipocytes are responsible for the higher levels of adipokines detected in the CM.

Increased expression of these adipokines in the adipocytes and a high secretion of them to the microenvironment around the adipocytes can contribute to systemic chronic low-grade inflammation; this kind of inflammation can trigger several tumorigenic signaling transduction pathways promoting tumor initiation and progression [30]. However, it will be necessary to reveal whether these specific adipokines, or whether a combination of them might be responsible for chronic low-grade inflammation.

The undetectable levels of TNF-α can be explained by the fact that only 50% of the protein synthetic components are found in the adipose tissue per se, the other 50% come from non-adipocyte cells such as immunocytes, vascular, stromal and other cells [13,31]. In the case of TNF-α, macrophages are the major source of TNF-α produced by white adipose tissue [32]. Moreover, Crawford et al (1997) found that the amount of TNF-α released by unstimulated adipose tissue *in vitro* is low [33].

Given that there is a direct correlation between leptin and cancer [34] and leptin is the main adipokine secreted from the adipocytes (especially from SA) [13], we expected to get a higher level of leptin in CM collected form co-cultured cells. Surprisingly, in contrast to the other adipokines, the fold change in the level of leptin was lower in the co-cultured cells comparing to the control cells. Gottschling--Zeller et al (1999) showed that the secretion of leptin from adipocytes can be suppressed by TGF-β [35] a growth factor, encoded by TGFB1 gene, which is over expressed in patients with colorectal cancer [36]. Likewise, we showed that leptin level was higher in HCT116 cells as compared to HM-7 cells. Our results may suggest that leptin is not the main adipokine responsible to regulate the crosstalk between cancer cells and adipocytes. Moreover, it seems that cancer cells with low malignant ability, such as HCT116 cells, can affect leptin secretion from the adipocytes more than aggressive HM-7 cells. We demonstrate herein that the levels of IL-8, IL-6, and MCP-1 were much higher when adipocytes were co-cultured with HM-7 cells than when co-cultured with HCT116 cells. This suggests the importance of the malignancy level of the colon cancer cells in their crosstalk with the adipocytes. As far as we know this is the one of the few studies that shows the importance of the cancer staging in the aspect of crosstalk with adipocytes. The rationale behind our results may be based on the fact that HM-7, as compared to HCT116, is a very aggressive cell line, which secretes mucin and other mutagenic factors that may have significant effects on the secretory activity of the adipocytes. Several studies demonstrated indeed that there is a wide array of proteins and factors that can be expressed in metastatic tissue and are completely different to those expressed in the primary tumors [24]. Moreover, we assume that aggressive cancer cells with higher proliferation ability such as HM-7 can induce a higher hypoxic condition [37] as compared to HCT116, driving the adipocytes to secrete higher levels of adipokines [38].

Another interesting result is the stronger effect of SA than VA on adipokines levels, when co-cultured with cancer cells. This result was unexpected especially if we consider the known harmful metabolic effects of VA [39,40]. However, some recent reports support our unexpected results. Klimčá kova et al [41] showed that in relation to obesity and metabolic complications, subcutaneous adipose tissue (SAT) acts similarly to the visceral adipose tissue (VAT). Their study showed that there is a substantial fraction of genes, which are expressed differently between the two depots, but still, the molecular adaptation, which is associated with obesity and metabolic syndrome, was as strong in SAT as in VAT [41]. Moreover, we showed that in the CM collected from co-cultured SA from obese subject and HM-7 the adipokines levels were significantly higher as compared to their levels in the CM collected from co-cultured SA from lean subject and HM-7, surprisingly we had the opposite trend in the VS results. We can explain unexpected result by looking to the adipocytes donor's

characteristics. As we presented in table 1, the donor of the lean visceral adipocytes had taken a medicine called Synthroid (manufactured form of the thyroid hormone thyroxine. It is used to treat thyroid hormone deficiency), studies showed that T3 (thyroid hormone) regulates adipogenesis and the related processes, lipogenesis and lipolysis *in vivo* as well as in cultures of adipocytes [42]. It is already known that visceral depot is more lipolytic than subcutaneous depot, so we assume that in this case of this donor, the consumption of this medicine raises the visceral fat lipolytic activity, leading to higher free fatty acids in the serum and eventually leading to higher insulin resistance. All the above can explain the reasons why our results in the adipocytes from lean donor was higher regarding to adipokines secretion and expression [39].

We detected in the present study additional metabolic effects of adipocytes on colon cancer cells. Firstly, FABP4 gene expression was elevated in HM-7 cells co-cultured with VA of lean or obese subjects. This is in line with Neiman et al (2011), who demonstrated that one of the mechanisms by which the cancer cells can proliferate and grow is by using fatty acids, provided by the surrounding adipocytes [24]. They tested the levels of FABP4 in the co-cultured cancer cells and the levels of HSL in the co-cultured adipocytes and found an up-regulation in the expression levels of both proteins.

However, we could not find similar effects in HM-7 cells co-cultured with SA, or in HCT116 cells. These finding is supported by results of Neiman et al (2011), demonstrating that FABP4 protein levels were low in primary omental tumors as compared to omental metastases [24]. Nonetheless, in our study we did not measure any change in HSL levels in the co-cultured adipocytes. We conclude that the crosstalk between HM-7 cells and VA from obese subject derived the cancer cells to express higher levels of FABP4 promoting the aggressive phenotype of HM-7 cells. It also can indicate a higher fatty acid transport in the cancer cells. However, the unchanged level of HSL in the adipocytes and the unchanged expression levels of CPT1 and ACOX1 genes in HM-7 cells indicate that HM-7 cells do not enhance the lipolytic pathway in the adipocytes, and that the fatty acids secreted by the adipocytes did not oxidize and been used as energy source to HM-7 cells.

Moreover, in an effort to understand how the higher levels of both the fatty acids and adipokines present in the CM, which are associated also to the high expression level of FABP4 gene in HM-7 cells, can affect the cancer cells metabolism, we conducted a bioenergetic experiment. We aimed at demonstrating if CM collected following different treatments will induce a bioenergetic effect in the cancer cells.

The increased oxidation phosphorylation process (OX-PHOS) reflected by the higher level of OCR and maximal OCR in the HM-7 cells after treating them with CM-F can be postulated to be measured due to the excess of fatty acids which supplied by adipocytes. These fatty acids are oxidized in the mitochondria through the OX-PHOS pathway. On the other hand, the adipokines abundance in the co-cultured CM (CM-F + HM-7) showed a small reduction in the OCR and maximal OCR compared to the CM-F; however, this reduction was not significant comparing to the control cells. These results can indicate that CM-F gives the cancer cells an advantage by increasing the OX-PHOS pathway, and by that achieving higher energy which is supplemented to the cells and increases their proliferation rate.

It is well known that obesity and fat accumulation are conditions, which combine oxidation stress. In cultured adipocytes there is increased secretion of fatty acids, these can induce increased levels of ROS and a higher oxidation stress, which can lead to an increase in the adipokines secretion [43]. We may assume that in CM-F + HM-7 the oxidation stress was much higher since the metastatic cells also produce higher levels of ROS [44]. Altogether, the higher levels of ROS and the adipokines secreted to the CM can induce a decrease in the escalating trend of the OX-PHOS pathway, diverting the cancer cells to use other metabolic pathways additional to OX-PHOS such as the pentose phosphate shunt promoting extensive growth, proliferation, and tumorigenesis of the cells [45].

To support our previous conclusion, we conducted invasion assay where the cancer cells were treated with the different CM. The invasion results show that CM-F + HM-7 of VS and SC adipocytes lead to an increase of 1.5 and 2.5 folds respectively in the invasion ability of the cells as compared to HM-7 cells treated with CM-F. The results highlight that there is indeed a crosstalk between the two cell populations and this crosstalk is necessary to induce the effect of invasion. Our results are supported by those of Dirat et al which showed the same trend [27].

Interestingly, the non-metastatic HCT116 cells react to oxidative stress or adipokines differently comparing to highly metastatic cells HM-7. When exposing HCT116 cells to CM-F + HCT116, OCR was significantly reduced compared to control cells. We assume that in HCT116 cells CM-F + HCT116 suppress the OX-PHOS pathway and instead the cells use another pathway to produce energy. Treating the cells with CM containing secretions of VS or SC adipocytes of obese subject and HCT116 cells (CM-F + HCT116); can induce increased proliferation of the cells leading them to use glutaminolysis which is an alternative metabolic pathway used in the malignant transformation process [37].

In summary, based on the co-culture system model, we can now highlight several novel conclusions:

1. Interaction between adipocytes and cancer cells promote pro carcinogenic effects in both cell types, regardless if they were adipocytes from obese or lean donor or if they were from visceral or subcutaneous depot.
2. Crosstalk between colon cancer cells and adipocytes affects mainly more malignant cells leading to the expression of several molecules directly associated to the malignant phenotype.
3. SC adipocytes from obese subject act as major mediators of colon cancer phenotype. However, it seems like VS adipocytes of obese subjects affect the HM-7 cells phenotype but are less affected by HM-7 presence.
4. We assume that in HCT116 and HM-7 colon cancer cells, glutaminolysis and pentose phosphate shunt additional to OX-PHOS may be the pathways that these cancer cells use to produce energy, leading them to gain a highly malignant phenotype.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.07.010.

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Disclosure of Potential Conflicts of Interest

The authors disclose no potential conflicts of interest.

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