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Unlocking the paracrine crosstalk: adipocyte-derived factors affect carbonic anhydrase IX expression in colon and breast cancer cells

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Obesity is a major public health concern because it increases the risk of several diseases, including cancer. Crosstalk between obesity and cancer seems to be very complex, and the interaction between adipocytes and cancer cells leads to changes in adipocytes' function and their paracrine signaling, promoting a microenvironment that supports tumor growth. Carbonic anhydrase IX (CA IX) is a tumor-associated enzyme that not only participates in pH regulation but also facilitates metabolic reprogramming and supports the migration, invasion, and metastasis of cancer cells. In addition, CA IX expression, predominantly regulated via hypoxia-inducible factor (HIF-1), serves as a surrogate marker of hypoxia. In this study, we investigated the impact of adipocytes and adipocyte-derived factors on the expression of CA IX in colon and breast cancer cells. We observed increased expression of *CA9* mRNA as well as CA IX protein in the presence of adipocytes and adipocyte-derived conditioned medium. Moreover, we confirmed that adipocytes affect the hypoxia signaling pathway and that the increased CA IX expression results from adipocyte-mediated induction of HIF-1α. Furthermore, we demonstrated that adipocyte-mediated upregulation of CA IX leads to increased migration and decreased adhesion of colon cancer cells. Finally, we brought experimental evidence that adipocytes, and more specifically leptin, upregulate CA IX expression in cancer cells and consequently promote tumor progression.

Key words: carbonic anhydrase IX; hypoxia; adipocytes; co-cultivation; leptin

Obesity poses a burgeoning challenge, impacting approximately 650 million adults globally [https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight]. In addition to its implications for cardiovascular health and metabolic dysfunction, obesity significantly increases the susceptibility to various malignancies, e.g., cancer of the colon and rectum, breast, kidney, endometrium, and pancreas [1]. Furthermore, it also acts as an unfavorable prognostic factor for numerous cancers, increasing the risk of cancer-specific mortality by 17% [2]. Besides promoting tumorigenesis, obesity complicates the treatment of already established cancers by altering the pharmacokinetics and pharmacodynamics of administered anti-cancer drugs [3] and, moreover, by modulating the tumor microenvironment (TME) [4].

Within the TME, adipocytes serve as an energy provider and metabolic regulator to promote the growth and survival of cancer cells. Moreover, adipocytes located close to invasive cancer cells, referred to as cancer-associated adipocytes (CAAs) [5], are crucial for breast as well as colon cancer development, progression, and resistance to antitumor therapy [6]. Several different mechanisms have been proposed to explain how adipocytes could alter the biological and molecular properties of cancer cells, including the secretion of adipokines, remodeling of the extracellular matrix, and metabolic changes [7]. Nowadays, adipokines secreted by adipocytes are considered key molecules linking obesity to cancer. Leptin, one of the most abundant adipokines, is known for its mitogenic, proinflammatory, anti-apoptotic, and proangiogenic effects [8]. Moreover, leptin expression is stimulated in adipocytes by low O_2 tension through transcriptional regulation by the hypoxia-inducible transcription factor (HIF-1) [9].

Hypoxia has a major role in the metabolic reprogramming of cancer cells and is therefore considered a hallmark of cancer. HIF-1 mediates the induction of adaptive transcrip-

tional changes and activation of ion transport machinery, endowing cancer cells with advantages in terms of migration, invasion, metastasis, and resistance to therapy [10]. HIF-1 is a heterodimer consisting of the oxygen-dependent α subunit and the constitutively expressed β subunit. Under normoxic conditions, HIF-1α undergoes proteasome degradation executed by the von Hippel-Lindau tumor suppressor protein (pVHL) [11]. Conversely, in hypoxia, HIF-1α escapes degradation, translocates into the nucleus, and after forming a heterodimer with HIF-1β, it activates a multitude of hypoxia-inducible genes that are involved in angiogenesis, erythropoiesis, cell proliferation and viability, glycolysis, pH regulation, and cell adhesion. HIF-1 targets include genes encoding mediators of angiogenesis such as vascular endothelial growth factor (VEGF) and VEGF receptors, enzymes of the glycolytic pathway such as hexokinase 2, lactate dehydrogenase (LDH), and glucose transporters (GLUT-1, GLUT-3), as well as carbonic anhydrase IX (CA IX) [12, 13].

In the context of adaptive responses within TME, the transmembrane enzyme CA IX emerges as one of the central players. CA IX belongs to the most strongly hypoxia-induced proteins, serving as an established indicator of chronic hypoxia [14]. Through its catalytic activity, CA IX reversibly converts carbon dioxide to bicarbonate ions and protons and therefore participates in pH regulation. CA IX activity contributes to the maintenance of an intracellular pH favorable for cancer growth and survival, and simultaneously it facilitates the acidification of extracellular pH, thereby promoting not only invasion and metastasis but also immunosuppression and resistance [15–19]. Aside from pH regulation, CA IX modulates E-cadherin-mediated cell adhesion via interaction with β-catenin [20]. The participation of CA IX in diverse aspects of cancer development and progression was summarized by Pastorekova and Gillies [21]. Because CA IX is rarely expressed in normal cells within the human body, and in contrast, highly expressed in hypoxic tumors, it has received considerable attention as an attractive biomarker and cancer-specific therapeutic target [22].

Adipocytes are considered to be important promoters of tumorigenesis in both colon and breast cancer. They store and secrete fatty acids (FAs) and adipokines and, thus have the potential to affect neighboring cells, particularly cancer cells, by paracrine and endocrine mechanisms. As CA IX is one of the most prominent hypoxia-regulated genes with an ultimately relevant role in tumor physiology, we decided to investigate the effect of adipocytes and adipocyte-derived factors on the expression of CA IX. In this study, we selected three different models of adipocytes (one human and two of mouse origin) and further analyzed the interplay between them and HCT116 and BT-20 cells derived from colon and breast cancer, respectively. We investigated the contribution of adipocytes to colon and breast cancer phenotype through elevated expression of CA IX and HIF-1α. We observed upregulation of *CA9* mRNA as well as CA IX protein following co-culture experiments with adipocytes and after treatment with adipocyte-derived conditioned medium. Furthermore, we provided direct proof that adipokine leptin is a novel regulatory molecule responsible for upregulating the expression of CA IX in cancer cells.

Materials and methods

Cell cultures. The human colon carcinoma cell line HCT116 (ATCC CCL-247) and breast carcinoma cell line BT-20 (ATCC HTB-19) were cultured in Dulbecco's modified Eagle's medium (DMEM, Biosera, LM-D1110/500, Cholet, France) or Minimum Essential Medium Eagle (EMEM, Lonza BioWhittaker, 12-662F, Walkersville, MD, USA), respectively. Medium was supplemented with 10% fetal calf serum (FCS, Biosera, FB-1001B/500), and 50 μg/ml gentamicin (Biowest, L0012-100, Nuaillé, France). Additionally, EMEM was also supplemented with 2 mM ultraglutamine (PanBiotech, P04-82100, Aidenbach, Germany). The HCT116 CA9 knockout cell line (HCT116 KO CA9) was generated using CRISPR/Cas9 technology, as stated in [23].

Preadipocytes 3T3-L1 (CL-173), kindly provided by Prof. Barbara Ukropcova (Institute of Experimental Endocrinology, BMC SAS), were cultured in DMEM containing 4.5 g/l glucose (Sigma-Aldrich, G7021, MO, USA) and 1.5 g/l $NaHCO_3$ (Sigma-Aldrich, S-5761). The medium was supplemented with 10% newborn calf serum (NCS, N4637, Sigma-Aldrich), 100 U/ml penicillin, and 100 U/ml streptomycin (1% P/S, Sigma-Aldrich, P4458). Adipogenic differentiation was induced according to the protocol of Zebisch et al. [24]. In brief, cells were seeded to reach full confluence in 2 d. After that, cells were differentiated using DMEM containing 10% FCS, 50 μg/ml gentamicin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, I5879), 0.25 μM dexamethasone (Sigma-Aldrich, D2915), 1 μg/ml insulin (Sigma-Aldrich, I9278), and 2 μM rosiglitazone (Sigma-Aldrich, R2408) for 2 d. Subsequently, differentiation medium was replaced with DMEM containing 10% FCS, 50 μg/ml gentamicin, and 1 μg/ml insulin. After 72 h, the medium was changed twice with DMEM with 10% FCS and 50 μg/ml gentamicin for the next 6 d.

Human multipotent adipose-derived stem cells (hMADS) were kindly provided by Dr. Ez-Zoubir Amri (Université Côte d'Azur, Institute de Biologie Valrose (iBV), Nice, France). Cells were maintained in low glucose DMEM (Biosera, LM-D1099/500) supplemented with 15 mM HEPES (Sigma-Aldrich, H0887), 10% FCS, 2 mM ultraglutamine, 2.5 ng/ml recombinant human FGF-2 (Peprotech, 100-18B), and 1% P/S. FGF-2 was omitted from the media after cells reached confluence. Adipogenic differentiation was induced by adding an adipogenic cocktail (1 μM dexamethasone, 500 μM IBMX, 10 μg/ml transferrin (Sigma-Aldrich, T8158), 10 nM insulin, and 0.2 nM triiodothyronine (Sigma-Aldrich, 709719) to DMEM/Ham's F12 medium (Lonza BioWhittaker, BE12-719F). 100 nM rosiglitazone was added to the media from day 2 to day 9. To obtain mature white adipo-

cytes, cells were kept in culture until day 18 in the absence of rosiglitazone.

All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2. Hypoxic experiments were performed in an anaerobic workstation (Ruskinn Technologies, Bridgend, UK) in a 1% O_2 and 5% CO_2 atmosphere.

Isolation of primary adipocytes. Primary mouse adipocytes were isolated from the subcutaneous adipose tissues of BALB/c mice. The adipose tissue was minced with scissors and incubated with 1 mg/ml collagenase II (Sigma-Aldrich, C6885) in collagenase buffer (25 mM $NaHCO_3$, 12 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.8 mM KCl, 120 mM NaCl, 1.4 mM $CaCl_2$, 5 mM glucose, 2.5% bovine serum albumin (BSA), and 1% P/S, pH 7.4) for 40 min at 37°C. The suspension was filtered through a 70 µm nylon filter (BD Falcon, 352350, NY, USA), followed by centrifugation at 1,000 × g for 10 min. The cell pellet was resuspended in DMEM containing 10% NCS and passed through a 40 µm nylon filter (BD Falcon, 352340). After centrifugation at 1,500 × g for 5 min, the cells were resuspended in DMEM containing 10% NCS and 1% P/S and seeded into 6-well plates. Adipocyte differentiation was induced by 2.4 nM insulin and 100 µM rosiglitazone to gain inguinal white adipocytes (iWAT). BALB/c mice were housed and used in accordance with the Institutional Ethics Committee guidelines under the approved protocols. The project was approved by the national competence authority-State Veterinary and Food Administration of the Slovak Republic (2101/19-221).

Production of conditioned medium. To produce conditioned medium (CM) from mouse (3T3-L1 or isolated from iWAT) and human (hMADS) adipocytes, cells were seeded in 6-well culture plates. Following adipocyte differentiation, fresh DMEM was added. After 24 h these media were centrifuged at 1,000 × g for 10 min at 4°C to remove any cellular debris and floating adipocytes. CM were then either used or stored at –80 °C.

Oil Red O staining. To visualize lipid droplets in adipocytes and cancer cells, Oil Red O (Sigma-Aldrich, O0625) staining was performed. Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min at RT. Following the fixation, cells were incubated with 60% isopropanol for 10 min, after which the Oil Red O staining was performed for 10 min. The lipid droplets were observed using light microscopy (Nikon Eclipse Ts2R, Tokyo, Japan). Spectrophotometric measurement was performed for quantitative analysis of lipid content in adipocytes and cancer cells. Bound Oil Red O stain was eluted with 100% isopropanol and quantified by measuring absorbance at 518 nm.

Fatty acid treatment. Oleic (Sigma-Aldrich, O1008) and palmitic acids (Sigma-Aldrich, P0500) were dissolved in ethanol at a 100 mM concentration and stored in aliquots at –20 °C. Prior to use, these fatty acids were conjugated to fatty acid-free BSA (Sigma-Aldrich, A7030) for 1 h at 37 °C and diluted in DMEM to a working concentration of 0.22 mM. Control cells were treated with BSA alone.

Leptin treatment. HCT116 and BT-20 cells grown in monolayer were first starved in DMEM containing 0.5% FCS. Following the starvation for 16 h, leptin (Sigma, L4146) was added at a concentration of 25, 50, or 100 ng/ml, and cells were incubated under normoxic or hypoxic conditions. After 24 h, cells were lysed for western blot. HCT116 spheroids were treated with 50 or 100 ng/ml leptin for 8 d.

Co-culture experiments. To establish a co-culture experiment, adipocytes (500,000) were seeded in the lower compartment of the Transwell system (Transwell inserts, SPL Life Sciences, 35006, Naechon-Myeon, Korea), while cancer cells (300,000) were seeded in the upper compartment. These two compartments were separated by a semi-permeable membrane with a pore size of 0.4 µm. Following a 24 h co-cultivation under normoxic or hypoxic conditions, total protein extracts or RNA were isolated and further analyzed.

Real-time PCR (qPCR). To determine the expression level of various genes in cancer cells, RNA was isolated using TRI Reagent™ Solution (ThermoFisher Scientific, AM9738, MA, USA) according to the manufacturer's protocol. 1 µg of RNA was used for the reverse transcription reaction done with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814). qPCR was performed using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, 172-5271, CA, USA) on a StepOne Real-Time PCR system (ThermoFisher Scientific). The procedure was as follows: 10 min at 95°C for initial denaturation, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Sample CT values were normalized to the housekeeping gene (β-actin). Relative expression was calculated using the ΔCT (ΔCT=2^–(CT gene of interest–CT housekeeping gene) method. All experiments were conducted in triplicates to ensure data reproducibility. The sequences of primers are as follows: *CA9* sense: 5'-ACTGCCTATGAGCAGTTGCT-3' and *CA9* antisense: 5'-TAGCCGAGAGTCACCAGGTC-3', *β-actin* sense: 5'-TCCTCCCTGGAGAAGAGCTA-3' and *β-actin* antisense: 5'-ACATCTGCTGGAAGGTGGAC-3', *LDHA* sense: 5'-TGGCAGCCTTTTCCTTAGAA-3' and *LDHA* antisense: 5'-ACTTGCAGTTCGGGCTGTAT-3', *GLUT1* sense: 5'-CAGAAGGTAATTGAGGAGTTCTACA-3' and *GLUT1* antisense: 5'-ACAAAGGCCAACAGGTTCATCATC-3'.

Western blot. Cells and spheroids were washed twice with PBS and lysed in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) supplemented with inhibitors of proteases (Sigma-Aldrich). Samples containing 30 µg of proteins were mixed with 2x Laemmli buffer and separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, gels were transferred to a nitrocellulose membrane (PVDF) for subsequent western blot (WB) analysis. Membranes were blocked with 5% non-fat milk in PBS and incubated with primary antibodies against CA IX (M75, 1:3, in-house hybridoma medium) [25], HIF1α (1:250; BD Biosciences, 610959, MA, USA), phospho-p44/42 MAPK (ERK1/2,

1:1000; Cell Signaling, #9101, MA, USA), phospho-mTOR (1:1000; Cell Signaling, #5536), and β-actin (1:5000; Cell Signaling, #3700). Following primary antibody incubation and washing steps, membranes were incubated with polyclonal goat anti-mouse or anti-rabbit immunoglobulins/HRP (P0447 or P0448, DAKO, CA, USA) 1:5000 in 5% non-fat dry milk for 1 h, RT. Protein bands were visualized using chemiluminescence (ECL) reagents on X-ray films.

Adhesion assay. HCT116 wild-type and HCT116 KO CA9 cells were pre-treated with CM or control medium for 24 h in normoxia. Subsequently, the cells were re-seeded in a 96-well plate in heptaplicates (50,000 cells/well). Medium containing unattached cells was removed after 1 h, adhered cells were washed with PBS, and after an additional 4 h incubation, adhesion was evaluated by MTT assay according to the manufacturer's protocol and measured at 570 nm.

MTT assay. Cells were seeded in a 96-well plate (22,000 cells/well) in octaplicates and incubated under normoxic or hypoxic conditions. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, M2128) assay. Following incubation with MTT for 2 h at 37°C, isopropanol was added for another 1 h. The absorbance was measured by a microplate reader (iBioTech Instruments, S1LFTA) at 570 nm.

Migration. HCT116 cells were starved in DMEM containing 0.5% FCS for 24 h and treated with CM. The cell migration process was assessed using the xCELLigence RTCA DP instrument (Agilent, CA, USA) in a 16-well CIM-plate 16 (Agilent, 5665817001) according to the manufacturer's instructions. The cells were seeded in octaplicates in the upper chamber (60,000 cells/well) in 0.5% DMEM or CM. The migration occurred through a microporous membrane into the lower chamber filled with 10% FCS DMEM or CM. The microelectrode sensor recorded alterations in electric impedance during cell migration. This real-time measurement provided the migration capacity of cells as a dimensionless variable known as the cell index.

Spheroid formation. Ultra-low attachment plates (BIOFLOAT™ ULAP, faCellitate, Manheim, Germany) were used for the formation of spheroids. HCT116 cells (1,000/well) were seeded either alone or in combination with differentiated 3T3-L1 adipocytes (1,000/well). After formation (5 d), the resulting spheroids were treated either with standard medium or CM for 8 d. Spheroids were incubated at 37°C with 5% CO_2, with medium exchange performed every 2 d. Spheroid formation was examined with a Zeiss Axiovert 40CFL microscope (Zeiss, Jena, Germany), and the size of spheroids was measured using ImageJ [26].

Clonogenic assay-colony formation assay. After 8 d of 3T3-L1 CM treatment, the spheroids were trypsinized, counted, and plated (200 cells/well) into adherent cell culture dishes. Subsequently, the cells were cultured for two weeks under normoxic conditions in a standard cultivation medium. The surviving colonies were fixed and stained with 0.25% crystal violet in ethanol.

Immunohistochemistry. Spheroids were embedded in paraffin according to the standard histological procedure. Paraffin-embedded spheroid sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using the Dual Endogenous Enzyme Block (DAKO, S2003) for 10 min. Following the protein block, sections were incubated with the primary antibody M75 (1:100) for 1 h in a humidified chamber. In the next step, sections were probed with EnVision+ System-HRP Labeled Polymer Anti-mouse (DAKO, K4001) for 30 min. Finally, immunoreactivity was visualized with the Liquid DAB+ Substrate Chromogen System (DAKO, K3468), and the sections were counterstained with EnVision FLEX Haematoxylin (DAKO, K8008) and mounted. Images were acquired using a bright-field microscope (Nikon Eclipse, Tokyo, Japan) with a Nikon Plan Fluor camera.

Flow cytometry. FA-treated and untreated HCT116 cells (400,000) together with the media were collected, washed twice with PBS, and stained with Zombie Violet (1:500, BioLegend, 77477, CA, USA) for 20 min in the dark at RT. The negative control was incubated with PBS alone. Subsequently, the cells were washed and analyzed. Flow cytometric analysis was performed using NovoCyte Advanteon (Agilent) with violet laser (405 nm). Viable and dead cell populations were distinguished based on Zombie Violet fluorescence intensity, with viable cells exhibiting low staining intensity and dead cells displaying high staining intensity. The data were evaluated using the NovoExpress software version 1.6.2 (Agilent).

Enzyme-linked immunosorbent assay (ELISA). Shedding of CA IX ectodomain (ECD) was analyzed by the DuoSet® ELISA Development System for Human Carbonic Anhydrase IX (R&D Systems, DY2188, MN, USA) according to the manufacturer's recommendations. CA IX ECD concentration (pg/ml) was determined by comparing absorbance values to a standard curve.

Statistical analysis. All data are presented as a mean ± standard deviation (SD). A two-tailed Student's t-test, or two-way ANOVA test, was used for statistical analysis. The statistical analysis was performed using GraphPad Prism, version 9 (GraphPad, La Jolla, CA, USA). A p-value < 0.05 was considered significant.

Results

Adipocyte-derived conditioned medium influences the expression of CA IX in colon cancer cells. To investigate the impact of adipocyte-secreted factors on breast and colon cancer cells, we used a conditioned medium (CM) produced by mouse 3T3-L1 adipocytes. Firstly, 3T3-L1 cells were seeded and differentiated according to the standard protocol. The differentiation was confirmed and visualized using Oil Red O staining. The 24 h medium from fully differentiated 3T3-L1 adipocytes was used for the treatment of cancer cells.

Cells treated with CM for 24 h in parallel with control cells (treated with fresh medium) were subsequently analyzed through WB and qPCR. While 3T3-L1 CM induced a significant increase of CA IX in normoxia, a clearly visible decrease was observed in hypoxia (Figure 1A). CM-induced expression of CA IX was revealed not only in colon (HCT116) but also in breast (BT-20) cells cultivated under normoxic conditions (Supplementary Figure S1). In line with the protein induction, CM treatment resulted in a significant increase of *CA9* transcription (>9-fold) in normoxia and its significant decrease in hypoxia ($p<0.001$) (Figure 1A). In addition to CA IX, the level of the alpha subunit of the HIF-1 transcription factor was also affected by 3T3-L1 CM treatment in both HCT116 (Figure 1A) and BT-20 (Supplementary Figure S1) cells. There were no discernible differences in the viability of HCT116 cells following 3T3-L1 CM treatment in normoxia, however, a significant decrease was observed in hypoxia (Figure 1A).

In order to confirm the effect of CM from 3T3-L1 adipocytes on CA IX expression, we employed additional adipocyte cell models of human (hMADS) and mouse origin (primary mouse adipocytes isolated from the inguinal white adipose tissue-iWAT). As in the previous case with 3T3-L1 CM, HCT116 cells were treated either with iWAT CM or hMADS CM in normoxia and hypoxia for 24 h. As expected, significantly increased *CA9* mRNA and CA IX protein expression were revealed in normoxia after iWAT CM (Figure 1B) or hMADS CM (Figure 1C) treatment. Conversely, the downregulation of *CA9* (Figures 1B, 1C) in line with the decreased level of HIF-1α observed in

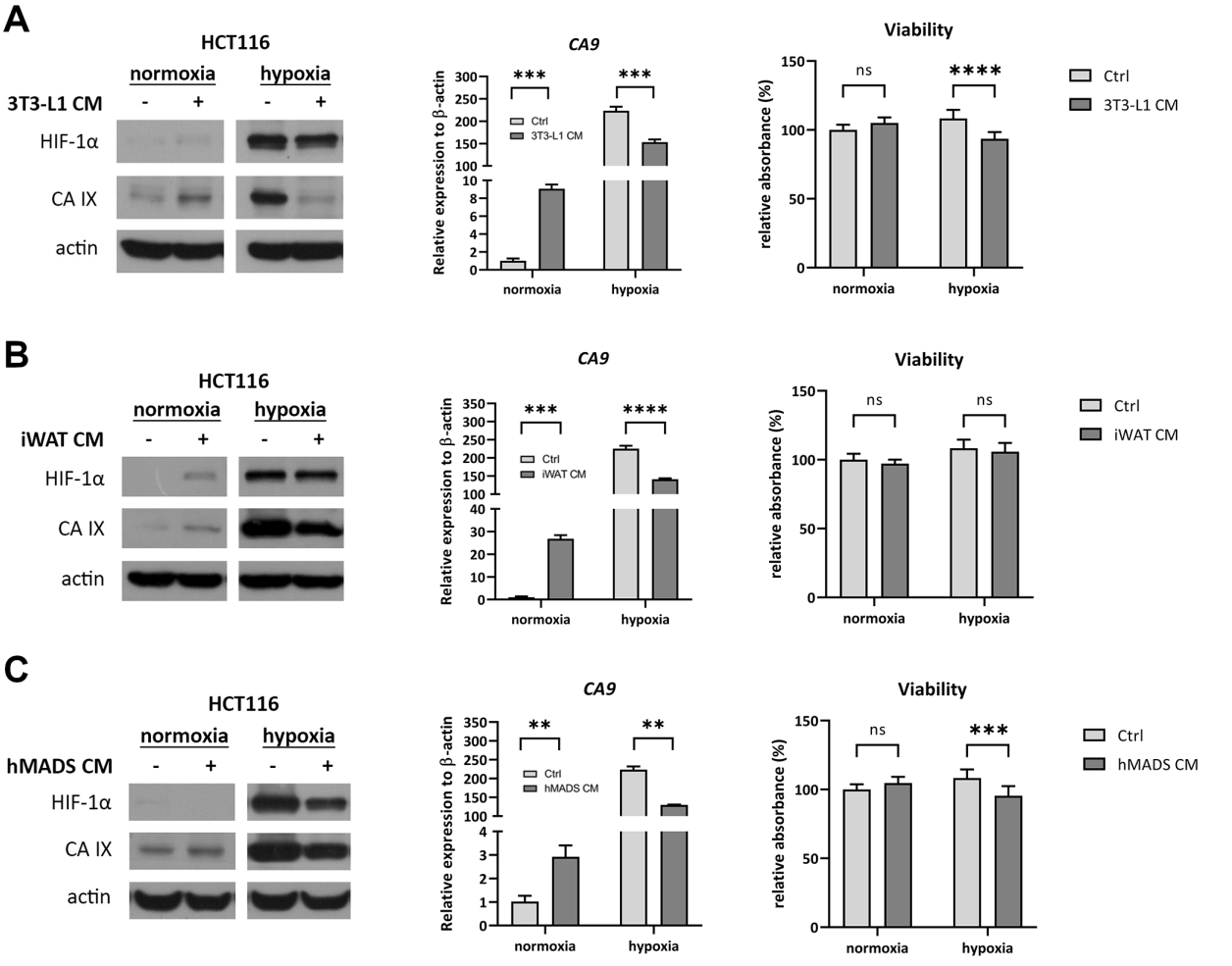

Figure 1. Conditioned medium (CM) from 3T3-L1 (A), iWAT (B), and hMADS (C) adipocytes affects the expression of CA IX protein and *CA9* gene. Western blot analysis depicting CA IX and HIF-1α levels with or without 3T3-L1 (A), iWAT (B), and hMADS (C) CM treatment. HCT116 cancer cells were incubated in normoxia or hypoxia for 24 h. Quantitative real-time PCR results from HCT116 cells treated either with fresh medium (Ctrl) or with conditioned medium from 3T3-L1 (A), iWAT (B), and hMADS (C) adipocytes incubated in normoxia or hypoxia for 24 h. The expression of *CA9* mRNAs was analyzed and normalized to *β-actin* levels. CM-treated values are expressed as the fold induction of the control ones. The significance of normoxia and hypoxia was analyzed separately. The viability of either control (Ctrl) or CM-treated HCT116 cells incubated in normoxia or hypoxia for 24 h was analyzed using an MTT assay. Data are presented as means ± SD. n.s. $p\geq0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$

hypoxia suggests that adipocyte-derived factors in CM can affect the overall response of cancer cells to hypoxia and the HIF-1-driven signaling pathway. Taken together, the effect of adipocyte-derived factors on *CA9* gene and CA IX protein expression and tumor cell viability seems to be conserved, as we utilized three different adipocyte cell models of both mouse and human origin, and consistent despite the different culture conditions of individual adipocyte *in vitro* models.

Adipocyte-derived conditioned medium influences adhesion, migration, and clonogenic potential of colon cancer cells. The abovementioned stimulating effect of adipocyte-derived conditioned medium on the expression of CA IX prompted us to investigate the biological properties of colon cancer cells regarding CA IX. It is well known that CA IX plays an important role in pH regulation, which is critical for the survival of cancer cells in hypoxia and acidosis. Moreover, CA IX is an active component of the cell migration machinery, where it functions through the control of cell adhesion and pH regulation at the leading-edge membranes of the migrating cells [20, 17].

Therefore, we analyzed the impact of the adipocyte-derived medium on the adhesion and migratory capacities of the HCT116 cells. In order to assess the role of CA IX in this process, HCT116 cell line with a knocked-out *CA9* gene (HCT116 KO CA9), prepared by CRISPR/Cas9 genome editing technology, was included in the adhesion assay. As shown in Figure 2A, 3T3-L1 CM treatment resulted in significantly decreased adhesion of HCT116 wild-type cells. On the contrary, no difference in adhesion capacity was observed in HCT116 KO CA9 cells after 3T3-L1 CM treatment, suggesting that the effect on decreased adhesion of HCT116 cells is directly mediated by the CA IX protein and its increased expression in response to 3T3-L1 CM treatment.

Following the adhesion assay, the migration capacity of HCT116 cells pre-treated with 3T3-L1 CM for 24 h was monitored using the xCELLigence system. As expected, normoxic HCT116 cells treated with CM exhibited increased migration compared to control cells treated with fresh medium (Figure 2B). Conversely, decreased migration of

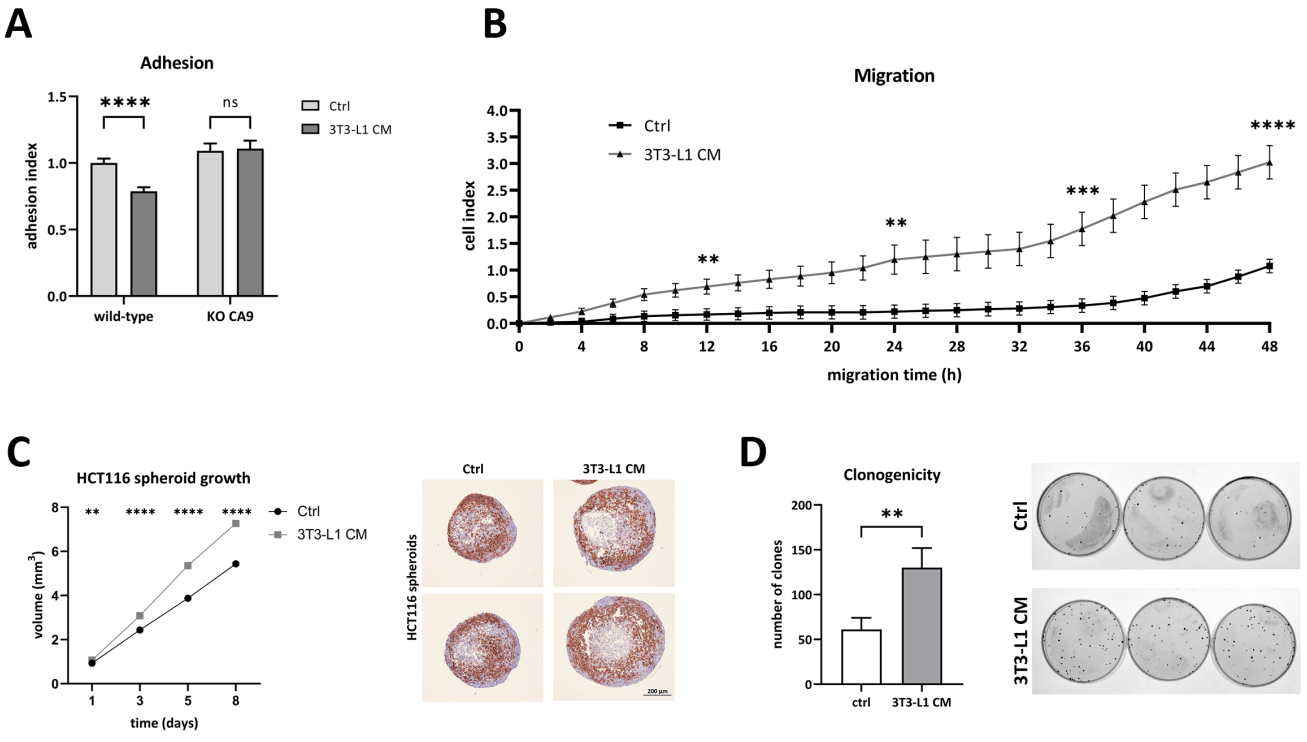

Figure 2. Adipocyte-derived CM reduces cell adhesion, stimulates cell migration, and increases the clonogenic potential of colon cancer cells. A) Graph representing the adhesion of HCT116 wild-type (wt) and HCT116 KO (knock-out) CA9 cells treated either with 3T3-L1 CM or fresh medium (Ctrl) for 24 h. The adhesion rate was assessed by an MTT assay. B) The migration ability of HCT116 cells, treated either with 3T3-L1 CM or fresh medium (Ctrl) in normoxia, was analyzed using the xCELLigence Real-Time Cell Analyzer. Cells were added in octaplicates to the upper chambers of the CIM-plate. Migration was expressed as a cell index representing a relative change of impedance monitored every 15 min for 48 h. C) The growth of HCT116 spheroids treated either with 3T3-L1 CM or fresh medium (Ctrl) was monitored by light microscopy. The size of spheroids was measured using ImageJ and was expressed as a volume in mm³. Representative images of the immunohistochemical staining of CA IX in spheroids treated with CM (3T3-L1 CM) or control spheroids (Ctrl) for 8 d. D) Colony forming assay of HCT116 cells derived from spheroids treated with 3T3-L1 CM. Following the 8 d treatment, the spheroids were trypsinized, plated, and incubated under normoxic conditions. After 14 d, colonies were stained with crystal violet and counted. Representative images of stained colonies are shown. Data are presented as means ± SD. n.s. p≥0.05; **p<0.01; ***p<0.001; ****p<0.0001

3T3-L1 CM-treated HCT116 cells was observed under hypoxic conditions (Supplementary Figure S2), which is consistent with the above results describing decreased CA IX expression after 3T3-L1 CM treatment in hypoxia. Taken together, these data indicate that adipocyte-derived factors affect CA IX expression, leading to decreased adhesion and increased migration, thus resulting in an increased metastatic potential of HCT116 colon cancer cells.

Three-dimensional cultures like spheroids are able to better control and more accurately mimic some features of solid tumors, e.g., generation of hypoxic cores with a gradient of oxygen and nutrients, which occur in the tumor microenvironment. As CA IX is an important component of the TME, we decided to employ a 3D spheroid model of cancer cells and subsequently study the effect of adipocyte-derived CM on clonogenic potential. For these purposes, we prepared HCT116 spheroids, either treated or untreated with 3T3-L1 CM. As opposed to the short-term treatment of monolayers, these spheroids enabled us to examine the prolonged effect of adipocyte-derived CM on cancer cells. The growth of spheroids was regularly monitored using a Zeiss Axiovert microscope and measured using ImageJ. The medium was exchanged every other day. Significantly enhanced growth of 3T3-L1 CM-treated HCT116 spheroids was observed during the whole treatment time (Figure 2C). In line with the increased spheroid size, we observed a more obvious necrotic region in HCT116 spheroids after CM treatment (Figure 2C). Following the 8 d, the spheroids were trypsinized and an equal number of cells from either control or CM-treated spheroids were cultivated for additional two weeks in a standard medium. As shown in Figure 2D, the clonogenic potential of cells derived from spheroids treated with 3T3-L1 CM was significantly upregulated compared to control spheroids treated with fresh medium, suggesting an additional supportive effect of adipocyte-derived CM on the survival of colon cancer cells., e.g., via increased availability of energy substrates.

Fatty acids affect the expression of CA IX in colon and breast cancer cells. Our above-presented experiments confirmed the supportive effect of adipocyte-derived conditioned medium on the biological properties (migration, adhesion, and clonogenic potential) of colon cancer cells, which correlates with increased CA IX expression in normoxia. However, the decreased expression of CA IX observed in CM-treated colon (Figure 1) as well as breast (Supplementary Figure S1) cancer cells in hypoxia was unexpected and sparked our curiosity.

Adipocytes are lipid-rich cells that primarily store long-chain fatty acids (FAs) in the form of triglycerides in lipid droplets. Interestingly, these FAs can be released and provided to cancer cells and consequently used as an energy supply. Such lipid transfer from adipocytes to cancer cells has emerged as a hallmark of malignancies thriving in a lipid-rich tumor environment [28], and therefore, we investigated whether and how FAs released from adipocytes could affect cancer cells, particularly CA IX expression. Firstly, we were interested whether hypoxic incubation for 24 h could affect lipid accumulation in mouse and human differentiated adipocytes. Oil Red O staining was examined by a microscope and then the quantitative analysis of lipid content in adipocytes was performed. As shown in Figure 3A, a decline in lipid content was revealed in all three adipocyte models after hypoxic incubation ($p<0.05$ in the case of 3T3-L1 and iWAT adipocytes; $p=0.149$ in the case of hMADS).

To confirm that CM-treated colon cancer cells are able to accumulate the lipids, HCT116 cells were treated with iWAT CM for 24 h. Following incubation in normoxia and hypoxia, Oil Red O staining was performed, and lipid droplets were examined in CM-treated cells as well as control cells treated with fresh medium. Interestingly, 24 h incubation of HCT116 cells with iWAT CM in hypoxia resulted in a clearly visible lipid accumulation in comparison to control HCT116 cells (Figure 3B), suggesting that colon cancer cells could hijack lipids released by adipocytes. Moreover, an elevated lipid accumulation observed in hypoxia could interfere with the hypoxia-induced signaling pathway and consequently with CA IX expression. To investigate whether the observed effects are mediated by FAs and their increased storage, we treated HCT116 cells with palmitic (PA) and oleic (OA) acids, the two most abundant saturated and monounsaturated fatty acids. FA-treated cells were analyzed in parallel with control cells (treated with BSA). Oil Red O staining examination not only confirmed the uptake of OA and incorporation to neutral lipids into HCT116 cells but also revealed a higher lipid accumulation in hypoxic cells in comparison to the normoxic ones (Figure 3C). Similarly, as in OA-treated cells, a clearly visible uptake of PA was observed in normoxia. However, decreased viability of PA-treated HCT116 cells was discovered in hypoxia (Figure 3D). Subsequently, FACS analysis with Zombie Violet enabled us to examine the viability of HCT116 cells after FA treatment. As expected, the viability of OA-treated cells under both normoxic and hypoxic conditions was comparable to that of the control cells (treated only with BSA). On the contrary, an increased proportion of dead cells (7.33% in normoxia and 25.68% in hypoxia) was revealed in HCT116 cells after PA supplementation (Figure 3D).

Next, we analyzed CA IX expression in both HCT116 and BT-20 cells after FA treatment. While a slight increase in CA IX expression after FA treatment was observed in normoxia (with OA in HCT116 cells and PA in BT-20 cells), a significant decrease after PA treatment was evident in hypoxia in both colon and breast cancer cells (Figure 3E). These data indicate that while increased expression of CA IX in normoxia could be induced by the uptake of FAs into cancer cells, the reduction of CA IX level could be caused by PA-induced lipotoxicity in cancer cells, especially under hypoxic conditions. In addition, increased shedding of the CA IX ectodomain, probably as a result of PA-mediated cell death, was observed in hypoxic HCT116 cells (Figure 3F). Altogether,

these data suggest that in normoxia, HCT116 cells could be affected by secreted factors (e.g., lipids) released from differentiated adipocytes, leading to increased CA IX levels (presented in Figures 1 and 3C) and enhanced metastatic potential (presented in Figure 2). On the contrary, decreased levels of HIF-1α protein and its downstream target CA IX after CM treatment could be, at least partially, explained by CM-mediated lipotoxicity.

Co-culture with adipocytes increases the expression of CA IX in colon and breast cancer cells. Numerous studies have elucidated the reciprocal crosstalk between cancer cells and adipocytes, primarily focusing on endocrine and paracrine signaling mechanisms. To determine the direct functional effects of adipocytes on cancer cells, a co-culture system was employed, effectively isolating cancer cells from adipocytes while facilitating the bidirectional exchange of secreted factors. Differentiated murine adipocytes (derived from the 3T3-L1 preadipocytes or isolated from iWAT), along with human adipocytes (hMADS), were subjected to a 24 h co-cultivation with cancer cells under normoxic and hypoxic conditions. A notable increase in the expression of CA IX was revealed after co-cultivation with 3T3-L1 adipocytes (Figure 4A). A stimulatory effect on CA IX protein was observed in both colon (HCT116) and breast (BT-20)

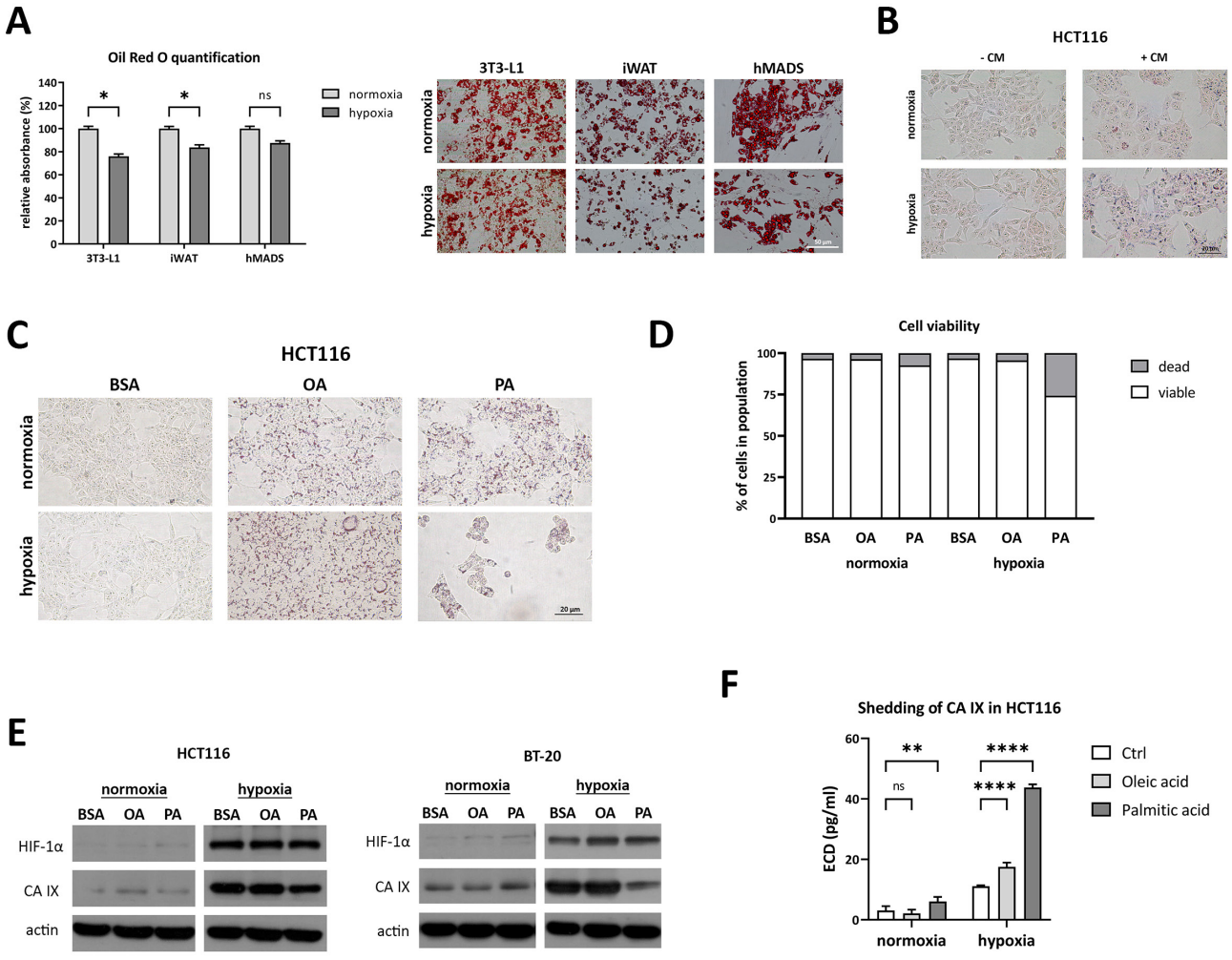

Figure 3. Fatty acid (FA)-mediated effects on the expression of CA IX and the viability of cancer cells. A) Oil Red O staining of differentiated 3T3-L1, iWAT, and hMADS cultivated under normoxic and hypoxic conditions for 24 h. Following light microscopy examination of lipid droplets, lipid content was quantified by spectrophotometric measurements and expressed as a percentage of hypoxic results in comparison to the normoxic values. B) Oil Red O staining of lipid droplets in HCT116 cells acquired after iWAT CM-treatment in normoxia and hypoxia for 24 h. C) Oil Red O staining of HCT116 cells after the treatment with either oleic (OA) or palmitic acid (PA) in normoxia and hypoxia for 24 h. Control cells were treated with BSA. D) Flow cytometry analysis with Zombie Violet measuring the viability of HCT116 cells following 24 h treatment with OA and PA acid. E) Western blot analysis of CA IX and HIF-1α expression in HCT116 and BT-20 cells after 24 h treatment with OA and PA acid. F) Shedding of CA IX ectodomain (ECD) investigated in control (BSA-treated) cells in comparison to cells treated with either OA or PA for 24 h. CA IX ectodomain (pg/ml) presented in the cultivation medium was quantified using an ELISA kit. Data are expressed as means ± SD. n.s. p≥0.05; *p<0.05; **p<0.01; ****p<0.0001

cancer cells. Moreover, the effect on co-cultivated cancer cells was comparable between 3T3-L1 and hMADS adipocytes (Figures 4A, 4B). Co-cultivation with iWAT adipocytes resulted in increased expression of CA IX in normoxia, however, this effect was not observed under hypoxic conditions (Figure 4B). Immunoblotting further revealed an upregulation of the HIF-1α subunit in hypoxia after 3T3-L1 and hMADS co-cultivation, indicating HIF-1-driven upregulation of CA IX. In addition, an increase in the expression level of *CA9* and additional hypoxia-inducible genes (*LDHA* and *GLUT1*) was observed after co-cultivation with differentiated 3T3-L1 adipocytes (Supplementary Figures S3A, S3B). All these findings collectively suggest that the co-cultivation of cancer cells with mature adipocytes induces the expression of CA IX, implying a potential contribution of adipocytes in shaping TME.

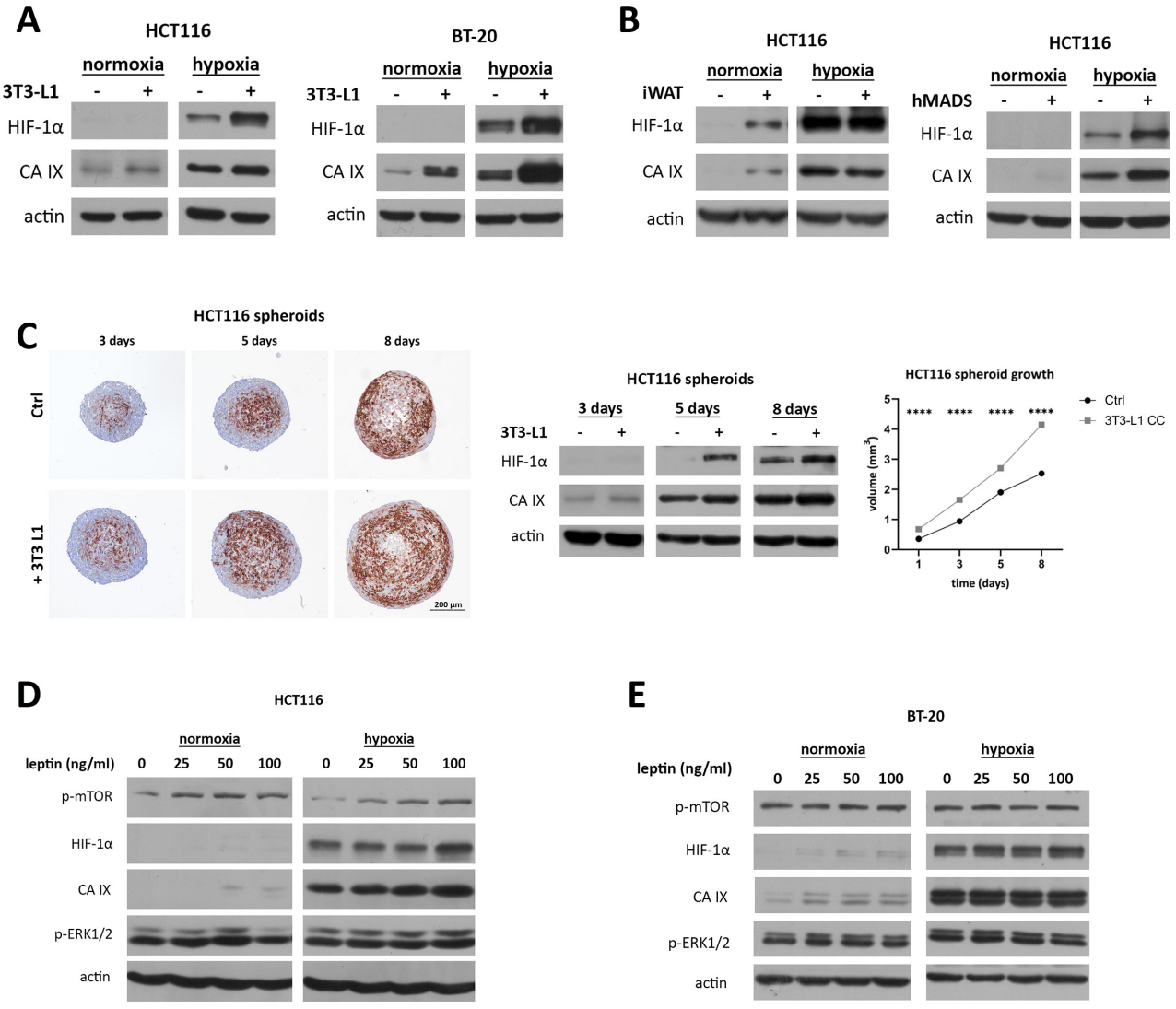

Figure 4. Co-cultivation with adipocytes (3T3-L1, iWAT, and hMADS) and leptin treatment increase the expression of CA IX in colon and breast cancer cells. A) Western blot analysis of CA IX and HIF-1α expression in HCT116 and BT-20 cells co-cultivated with differentiated 3T3-L1 adipocytes compared to control cells cultivated alone. Cells were co-cultivated under normoxic as well as hypoxic conditions for 24 h. B) Western blot analysis of HCT116 cells co-cultivated with either iWAT or hMADS adipocytes in normoxia and hypoxia for 24 h. C) Immunohistochemical analysis of CA IX expression pattern in homo- (HCT116 alone) as well as heterotypic (HCT116 mixed with differentiated 3T3-L1) spheroid analyzed after 3, 5, and 8 d. Western blot analysis of CA IX and HIF-1α expression in both types of spheroids. The growth of homo- and heterotypic spheroids was monitored by light microscopy. The size of spheroids was measured using ImageJ and was expressed as a volume in mm³. D) Western blot analysis of the effect of leptin treatment (25, 50, and 100 ng/ml) on the expression of CA IX, HIF-1α, p-mTOR, and p-ERK1/2 in HCT116 cells after 24 h. E) The effect of leptin treatment (25, 50, and 100 ng/ml) on the expression of CA IX, HIF-1α, p-mTOR, and p-ERK1/2 in BT-20 cells analyzed in normoxia ad hypoxia after 24 h. Data are expressed as means ± SD. ****p<0.0001

Next, we wanted to find out whether higher CA IX expression in colon cancer cells after co-cultivation with adipocytes could be observed in 3D spheroids. Therefore, we prepared co-cultured spheroids formed from an equal number of cancer cells and adipocytes. HCT116 cells (1,000/spheroid) were mixed with differentiated 3T3-L1 adipocytes (1,000/spheroid) and further analyzed at several timepoints (after 3, 5, and 8 d). Homotypic spheroids were formed only from 1,000 HCT116 cells/spheroid. The expression pattern of CA IX protein was examined by immunohistochemistry using the M75 antibody. As shown in Figure 4C, increased expression of CA IX was revealed in heterotypic spheroids formed from HCT116 and 3T3-L1 cells compared to homotypic spheroids. Moreover, an increased level of CA IX and HIF-1α proteins was revealed by WB, suggesting that the increased level of CA IX is linked with the hypoxic signaling pathway (Figure 4C). The growth curves presented in Figure 4C document a significantly faster growth of co-cultured spheroids in comparison to homotypic HCT116 spheroids. Although the initial number of cells in the heterotypic spheroids was higher than in the homotypic ones, it did not lead to an increased volume of spheroids since the differentiated adipocytes are no longer dividing. The presence of differentiated adipocytes within co-cultured spheroids promoted the growth of cancer cells, leading to their increased volume and, moreover, CA IX expression.

Besides fatty acids, adipocytes also release a whole spectrum of biologically active proteins termed adipokines, that could participate in other mechanisms underlying the interplay between adipocytes and cancer cells. Among them, leptin is one of the most abundant and most investigated. To examine the effect of leptin on CA IX expression, HCT116 cells were first starved and then treated with increasing concentrations (25, 50, and 100 ng/ml) of recombinant leptin in normoxia and hypoxia for 24 h. As shown in Figure 4D, elevated expression of HIF-1α and CA IX was revealed in leptin-treated HCT116 cells. Additionally, phosphorylation of ERK1/2 (p-ERK) and mTOR (p-mTOR) was substantially increased in leptin-treated cells, suggesting that, besides hypoxia, additional signaling pathways could participate in upregulation of HIF-1α and its downstream target CA IX in response to adipocyte-derived factors. In addition to the HCT116 monolayer, increased expression of CA IX and HIF-1α was revealed also in 3D spheroids treated with leptin for 8 d (Supplementary Figure S3C). To confirm that the effect of leptin on the expression of CA IX is not limited to colon cancer cells, we performed leptin treatment on BT-20 cells. As expected, an upregulated level of CA IX and HIF-1α was revealed in BT-20 cells after 24 h, but only in normoxia (Figure 4E). The already high induction of CA IX in response to hypoxia and HIF-1α activity could, at least partially, explain the fact that CA IX was not induced by leptin. Taken together, these data indicate a novel regulatory pathway responsible for increased CA IX expression in cancer cells and, moreover, provide evidence for a link between adipose tissue and cancer cells mediated by leptin.

Discussion

Tumor initiation and development are closely connected and ultimately tied up with the surrounding TME. Given the close relationship between obesity and tumor progression, adipocytes are considered to be crucial components of TME. Previous studies have identified alterations in adipocyte features upon exposure to cancer cells, resulting in their transformation into cancer-associated adipocytes (CAAs) [5, 29–31]. Such interplay between adipocytes and cancer cells could be particularly relevant at the invasive front of tumors where both cell types are in tight contact and where adipocytes could provide a wide array of effectors, e.g., adipokines, lipids, and exosomes [6].

In this study, we investigated the impact of adipocytes and adipocyte-derived factors on the expression of CA IX. To better explore the effect on CA IX, we analyzed colon as well as breast cancer cells. Firstly, we employed conditioned media from three different adipocyte models and examined their effect on cancer cells under normoxic and hypoxic incubation. As WAT represents the most prominent and dynamic adipose tissue in the human body, we decided to analyze this particular type. We used three white adipocyte models: hMADS, 3T3-L1, and primary iWAT adipocytes [32]. We revealed that CM derived from all three adipocyte models elevated *CA9* mRNA and CA IX protein expression in normoxic cancer cells. The effect was comparable between all three adipocyte models and was observed in both colon cancer and breast cancer cells. Considering the fact that CM-mediated induction of CA IX expression in normoxia could be at least partially caused by an increased density of cancer cells [33], we examined the proliferation of CM-treated cells. Importantly, we observed no significant differences in the proliferation of CM-treated HCT116 cells in normoxia after 24 h.

Previous studies reported that adipocytes and adipocyte-secreted factors could affect the biological properties (e.g., enhanced motility) and molecular characteristics of cancer cells (e.g., expression of genes associated with a more aggressive phenotype) [5, 31, 34, 35], although the nature of these factors is still under investigation. As mentioned before, CM-treated colon and breast cancer cells exhibited increased expression of CA IX in normoxia. To investigate whether adipocyte-derived medium also influences the functional characteristics regarding CA IX, we performed migration and adhesion assays. In line with our expectations, increased migration and decreased adhesion of 3T3-L1 CM-treated HCT116 cells were shown. Given no difference in adhesion capacity observed in HCT116 cells with knocked-out *CA9* gene after 3T3-L1 CM treatment, we confirmed that the effect on decreased adhesion of HCT116 cells is directly mediated by the CA IX protein and its

increased expression in response to 3T3-L1 CM treatment. Similarly, increased migration of colorectal cancer cells and, moreover, chick embryo chorioallantoic membrane angiopoiesis in response to the adipocyte-conditioned medium were reported by Xiao et al. [36].

Furthermore, we analyzed the efficiency of conditioned media to affect the clonogenic potential of HCT116 cells derived from 3D spheroids. Interestingly, the increased clonogenic potential was observed after prolonged treatment with 3T3-L1 CM. Enhanced clonogenic growth of breast cancer cells co-cultured with serum-activated normal fibroblast was reported by Samoszuk et al. [37]. It is not surprising that besides enhanced migration and invasion, adipocytes and adipocyte-derived CM could provide additional benefits to cancer cells, e.g., protection from the effects of anticancer drugs. Liu et al. demonstrated the adipocyte-mediated resistance of multiple myeloma cells to various chemotherapeutics [38]. Similarly, 3T3 and hMADS-derived CM induce resistance of HER2-positive breast cancer cells to the tyrosine kinase inhibitor lapatinib [39].

It is well known that adipocytes are lipid-rich cells that primarily store long-chain fatty acids as neutral lipids in lipid droplets (LDs). FAs can be provided to cancer cells and upon their uptake, they can support proliferation as well as rapid growth of cancer cells, either as building blocks in their membranes or as energy sources supplying ATP via mitochondrial β-oxidation. Such lipid transfer from adipocytes to cancer cells mediated via FA translocase (CD36), FA-binding protein (FABP), and FA transport protein (FATP) family as reviewed in [40] has emerged as a hallmark of malignancies occurring in a lipid-rich TME [28, 41]. Especially for the CD36 transporter, its higher expression is closely associated with the protumorigenic phenotype and, moreover, an unfavorable prognosis across all cancer types [42].

Firstly, we compared the accumulation of LDs in differentiated adipocytes under both normoxic and hypoxic conditions. Interestingly, we revealed decreased lipid content in all three tested models of adipocytes after 24 h of incubation in hypoxia. Recently, hypoxia-driven lipolysis was observed in human and mouse adipocytes upon treatment with cancer CM [43]. In an elegant study by Iwamoto et al., hypoxia-induced lipolysis in adjacent adipose tissue and upregulated CD36 expression were reported after bevacizumab treatment, resulting in higher FA release and uptake in different cancer types (colorectal, hepatocellular, and pancreatic) [44]. Next, we confirmed higher lipid accumulation into HCT116 cells after iWAT CM treatment, which was more pronounced after incubation in hypoxia for 24 h. Similarly, Benseed et al. observed elevated FA uptake and lipid storage in hypoxic glioblastoma and breast cancer cells as a consequence of HIF-1α-mediated upregulation of FABP3 and FABP7 expression [45]. On the contrary, FAs were demonstrated to be independent regulators of HIF transcription factors [46]. Based on the abovementioned observations, we assume that a reciprocal interaction between hypoxia and FAs could at least partially explain our data acquired with adipocyte-derived CM under hypoxic conditions.

Fatty acids, either synthesized *de novo* or taken up from the surrounding microenvironment, are closely associated with the development and progression of cancer [47, 48]. The most abundant saturated and monounsaturated FAs in the Western diet are palmitic (PA) and oleic acid (OA), and therefore, we decided to examine their effect on CA IX expression in colon as well as breast cancer cells. Surprisingly, we observed elevated expression of CA IX protein in HCT116 and BT-20 cells in normoxia after OA and PA treatment, respectively. Additionally, we revealed slightly increased expression of HIF-1α upon OA treatment in both cancer types, suggesting that OA could affect the overall hypoxic response in cancer cells. Consistent with our observations, Seo et al. demonstrated an increased level of HIF-1α after OA treatment and suggested that the FA-induced FABP5/HIF-1 axis promotes lipid accumulation and cell proliferation in hepatocellular carcinoma cells [49]. Furthermore, OA was revealed to induce proliferation and promote matrix metalloproteinase 9 secretion and invasion in breast cancer cells [50]. However, in our experiments, the viability of HCT116 cells after OA treatment was not significantly affected, which is in line with previously published reports using breast cancer cells (MDA-MB-231) [51]. On the other hand, we observed that PA supplementation resulted in a clearly visible decrease in a viability of HCT116 cells and an increased shedding of CA IX ectodomain (ECD) under normoxic as well as hypoxic conditions. Such apoptosis-induced ECD shedding of CA IX was reported by Vidlickova et al. [52]. Similarly, previously published papers reported that PA treatment reduces the viability of breast cancer cells and inhibits prostate cancer cell proliferation and metastasis [53, 54]. Since lipotoxicity depends on the chain length and saturation of the FA molecule [55], PA supplementation could result in lipotoxicity, leading to the apoptotic death of the HCT116 cells in our experiments.

All our experimental data discussed so far have been performed on adipocyte-derived conditioned medium. As we considered the limitations of conditioned medium acquired from differentiated adipocytes in normoxia, we employed co-cultivation experiments to investigate the interplay between adipocytes and cancer cells under both normoxic and hypoxic conditions. The co-culture system enabled us to separate cell populations while facilitating the exchange of secreted factors. Data from several studies confirmed that the co-cultivation of adipocytes and cancer cells has impactful and fostering significant effects on breast cancer progression and thus confirmed the indispensable role of the co-culture system in adipocyte-cancer cell-related research [5, 34, 41, 56, 57]. Firstly, Dirat et al. showed increased invasive capacities *in vitro* and *in vivo* of murine and human cancer cells co-cultivated with mature adipocytes [5]. Additionally, the induction of EMT in breast cancer cells upon co-cultivation with adipocytes, resulting in heightened migration and invasion,

was revealed by Lee et al. [34]. Balaban et al. observed that adipocyte-derived FAs drive breast cancer proliferation and migration and, thus, support cancer progression [56]. The phenomenon of exploring adipocyte-cancer cell interactions through co-cultivation extends beyond breast cancer, as has been performed also with pancreatic [30], ovarian [58], prostate [59], colon [60], and melanoma [61] cancer cells.

Therefore, we investigated the effect of co-cultivation with 3T3-L1 adipocytes on CA IX under normoxic and hypoxic conditions. In comparison to mono-cultured HCT116 and BT-20 cells, upregulated expression of CA IX was revealed after co-cultivation with 3T3-L1 adipocytes. In line with our results with CA IX, higher expression levels of GLUT1 and GLUT3 in colon cancer cells (HCT116 and LS180) co-cultivated with 3T3-L1 adipocytes were observed by Olszanska et al. [31], however, their co-cultivation experiments were performed only in normoxia. In addition to 3T3-L1 adipocytes, we observed increased expression of CA IX after co-cultivation with additional adipocyte models iWAT and hMADS which have a more pronounced effect in normoxia and hypoxia, respectively. Very recently, Fei et al. published increased expression of CA IX and decreased expression of CA III in adrenocortical cancer cells after co-cultivation with human primary adipose cells (ASCs) [62]. Significant upregulation of CA IX was observed only in H295R cells but not in MUC-1 cells co-cultured with ASCs, and on the other hand, a markedly increased CA IX level was induced in ASCs after co-cultivation with H295R cells, suggesting a reciprocal interaction between adipocytes and cancer cells. Surprisingly, they revealed for the first time CA IX expression in visceral adipose tissue, however, with no substantial differences between lean and obese subjects. In line with this, we also confirmed CA IX expression in differentiated hMADS, which was upregulated under hypoxia, however, we were unable to detect it in murine adipocytes (data not shown). Nevertheless, our experimental design was not limited to normoxia but also included hypoxia often present in solid tumors, and therefore provided a more complex and relevant view of the crosstalk between adipocytes and cancer cells.

In addition to the co-culture system, we also examined the expression of CA IX in heterotypic spheroids formed from adipocytes and colon cancer cells. Heterotypic spheroids were employed not only to adequately mimic a direct interaction between 3T3-L1 adipocytes and HCT116 cancer cells but also to compare a prolonged interaction (for 8 d in spheroids) with a short-term interaction (co-cultivation for 24 h). Interestingly, a direct contact between both cell types mixed in heterotypic spheroids resulted in more pronounced upregulation of CA IX as well as HIF-1α expression in comparison to homotypic HCT116 spheroids. The effect was clearly visible during the whole time period. Nowadays, heterotypic spheroids composed of cancer cells and cells of the TME (mostly fibroblasts) are widely employed and analyzed in order to investigate the direct interaction of both types of cells in a physiologically relevant setting. Recently, an elevated expression of CCL5/CCR1 was demonstrated in heterotypic spheroids consisting of ASCs and breast cancer cells, which consequently led to enhanced migration of MDA-MB-231 cells [63].

Several studies have demonstrated the relevance of endocrine and paracrine signaling mechanisms enabling the crosstalk between adipocytes and cancer cells. One of the proposed mechanisms underlying the risk association is the adipocyte-mediated secretion of adipokines. Adipokines, acting locally or systemically, play important roles in the growth, local invasion, metastatic spread, and resistance to treatment of different types of cancer. Currently, hundreds of different adipokines have been identified, and among these, leptin is one of the most abundant and most investigated, especially in breast and colorectal cancer [64, 65]. Leptin activates Janus-activated kinase (JAK)-2 through the leptin receptor (ObR) and promotes signaling through effector cascades, including signal transduction and activators in the phosphatidylinositol 3-kinase (PI3K)/AKT and transcription activator (STAT)-3 pathways [66]. Previous studies have revealed that leptin treatment could induce VEGF expression in human and mouse mammary tumors [67, 68]. Interestingly, upregulated expression of leptin in preadipocytes and adipocytes was revealed under hypoxic conditions [69], and moreover, HIF-1-mediated regulation and activation of ObR were observed in pancreatic cancer cells [70]. Additionally, leptin production and secretion affect several aspects of cancer cells, e.g., proliferation, differentiation, self-renewal, metastasis, chemoresistance, and angiogenesis [71]. Considering the facts that leptin production is upregulated i) in mature adipocytes, ii) in response to hypoxia, and iii) upon co-cultivation of adipocytes with cancer cells [72], we were interested in revealing its effect on CA IX. Following the 24 h treatment of HCT116 cell with leptin, we observed a dose-dependent increase of CA IX expression. In addition to elevated HIF-1α, we revealed increased phosphorylation of the ERK1/2 and mTOR signaling pathways in response to leptin treatment. Besides HCT116 cells cultivated in monolayers, leptin-mediated induction of CA IX was also observed in HCT116 spheroids and BT-20 cells cultivated under normoxic conditions. In addition to the abovementioned effects of leptin on cancer cells, cancer metabolism can also be affected. As an example, mitochondrial respiration was reported to be suppressed by leptin in HCT116 cells [73], indicating a shift to the glycolytic phenotype. Recently published studies by Benej and Gibadulinova demonstrated several experimental data linking the pH-regulating activity of CA IX to glycolysis [74, 75], suggesting an additional argument supporting the relevance of leptin-induced expression of CA IX in cancer cells.

We were aware of some limitations, especially the short-term co-cultivation, which did not allow us to fully reveal the mutual effects of both cell types. Considering this fact, we employed a heterotypic spheroid model, which enabled us to confirm adipocyte-driven upregulation of CA IX as

well as HIF-1α expression after prolonged cell-cell interactions. Additionally, the majority of our results was generated using mouse 3T3-L1 adipocytes and 3T3-L1-derived CM in combination with human cancer cells, and one could argue that these data cannot be directly applicable to humans. However, the 3T3-L1 cell line represents a well-established and frequently used cell line with a homogenous response following treatments and therefore remains the most commonly used cell model for studying the adipogenic differentiation process as well as obesity and its co-morbidities [76]. Despite the aforementioned limitations, our study provides the most comprehensive overview of the effect of adipocytes and adipocyte-derived factors on the hypoxia signaling pathway and especially on CA IX.

In the last two decades, most attention has focused on the investigation of the crosstalk between adipose tissue and cancer cells and, moreover, on the identification of key molecules responsible for the link between obesity and cancer. However, the mechanisms mediating the effects of adipocytes and adipokines on cancer cells have not been fully clarified yet. In the present study, we provide experimental data about the interplay between adipose tissue and cancer cells regarding the hypoxia-signaling pathway and CA IX expression. Given that these cell types co-exist in a dynamic TME, we investigated their interaction under normoxic as well as hypoxic conditions. We revealed increased expression of CA IX in both colon and breast cancer cells upon exposure to adipocyte-derived medium or co-cultivation with mature adipocytes. We brought the first experimental evidence of leptin-induced expression of CA IX in colon and breast cancer cells. We anticipate that the data presented in this study will broaden our knowledge about the interplay between obesity and cancer and, consequently, contribute to the development of more effective therapeutic strategies for obesity-related malignancies.

Supplementary information is available in the online version of the paper.

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Unlocking the paracrine crosstalk: adipocyte-derived factors affect carbonic anhydrase IX expression in colon and breast cancer cells

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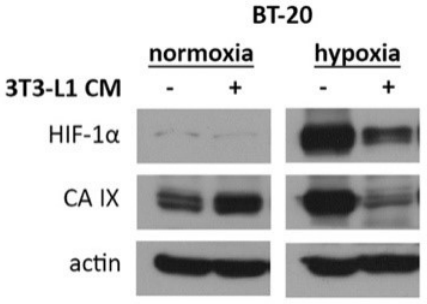
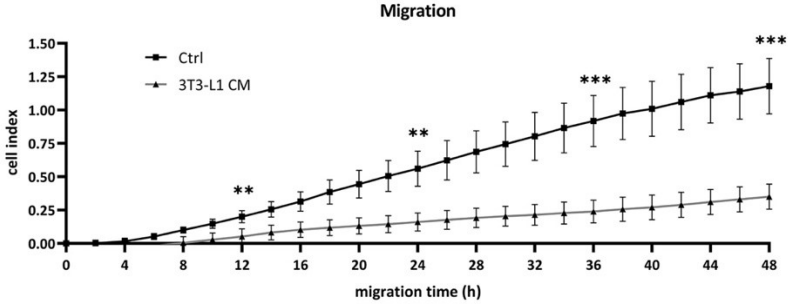
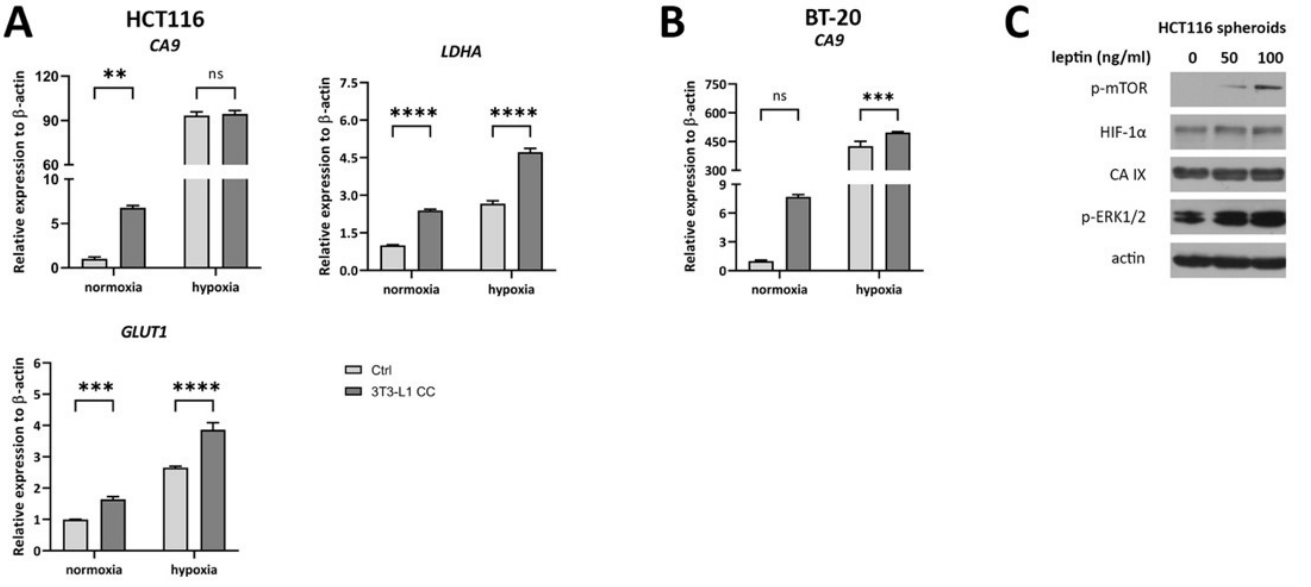
Supplementary Information



Supplementary Figure S1. Western blot analysis depicting CA IX and HIF-1α levels with or without 3T3-L1 CM treatment. BT-20 cancer cells were treated in normoxia or hypoxia for 24 h.



Supplementary Figure S2. Migration ability of HCT116 cells treated either with CM (3T3-L1 CM) or fresh medium (Ctrl) cultivated in hypoxia was analyzed using the xCELLigence Real-Time Cell Analyzer. Cells were added in octaplicates to the upper chambers of the CIM-plate. Migration was expressed as cell index representing the relative change of impedance monitored every 15 min for 48 h. **p<0.01; *** p<0.001



Supplementary Figure S3. Quantitative real-time PCR results from HCT116 (A) and BT-20 (B) cells co-cultivated with 3T3-L1 adipocytes. The expression of the *CA9, LDHA* and *GLUT1* gene was analyzed and normalized to *β-actin* levels. CC-values are expressed as the fold induction of the control ones. C) The effect of 8-day leptin treatment (50 and 100 ng/ml) on the expression of CA IX, HIF-1α, p-mTOR and p-ERK1/2 in HCT116 spheroids. Data are presented as means ± SD. n.s. p≥0.05; **p<0.01; ***p<0.001; ****p<0.0001