Secreted factors from adipose tissue reprogram breast tumor lipid metabolism and induce motility by modulating PPARα/ANGPTL4 and FAK

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Supplementary materials and methods

BrdU cell proliferation Assay

Cell proliferation was assessed using a BrdU Cell Proliferation Assay (Cell Signaling #6813) according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate (MDA-MB-231: 12000/ well; HCC38: 20000/ well) and incubated for 48h in cell culture medium. Cells were washed with DPBS and medium was changed to basal medium or ACM. After 24h, 10x BrdU solution was added and cells were incubated for an additional 16h (BrdU incorporation). Absorbance was measured at 450nm on a Multiskan Go (Thermo Fisher) spectrophotometer.

Invasion Assay

MDA-MB-231 and HCC38 cells (50000 cells/ well) were seeded in 24-well Matrigel Invasion Chambers (Corning #354480). Cell suspension was prepared in basal medium or ACM and transferred to the top well of invasion chambers. The corresponding plate well was filled with basal medium or ACM, respectively. Cells were incubated for 24h before cell invasion was quantified according to manufacturer´s instructions and as previously described (1). In brief, invaded cells were fixed with 100% methanol and cell nuclei were stained with DAPI (1µg/ml in PBS). Quantification was performed by photographing five fields per membrane at a magnification of 20x using a fluorescence microscope (Zeiss ApoTome). Cell invasion was calculated as the mean of invaded cells/ field.

Wound healing assay

The migration capabilities of MDA-MB-231 and HCC38 were analyzed by wound healing assay using Ibidi culture inserts (IBIDI #80209). Cells were seeded into both wells of the culture inserts (20000/ well) and incubated for 48h. After the cells had reached a confluency of 90-100%, the culture inserts were removed. Cells were washed with DPBS and treated with basal medium or ACM for 24h. The area of the wound was determined by phase contrast microscopy with 10x magnification (Zeiss Primovert). The relative wound closure was assessed by measuring the area of the wound at three distinct reference points before

(t=0h) and after the cell treatment with basal medium or ACM for 24 h (t=24h). Relative wound closure [%] represents the ratio of the wound area after 24 hours and the wound area at time zero.

siRNA knockdown of ANGPTL4

MDA-MB-231 cells were grown to a confluency of 60-70 %. Prior to siRNA transfection, cell culture medium was replaced by fresh DMEM/10% FBS. Cells were transfected using Lipofectamine RNAiMAX (Thermo Fisher #13778) according to manufacturer´s instructions (forward transfection). The used siRNAs for *ANGPTL4* knockdown, siANGPTL4_1 (Thermo Fisher # s27510), siANGPTL4_2 (Thermo Fisher # s27509) and for non-targeting control, siNTC1 (Thermo Fisher # 4390843) were complexed with the transfection reagent. The siRNA-lipid complex was added to the cells at a final siRNA concentration of 10nM. MDA-MB-231 cells were incubated with the siRNA-lipid complex for 24h, if not indicated otherwise. The efficiency of the knockdown was analyzed by qPCR.

Generation of stable ANGPTL4 depleted cells

Lentiviral knockdown of *ANGPTL4* was achieved using Dharmacon GIPZ Lentiviral particles (GE Healthcare) according to manufacturer´s instructions. MDA-MB-231 cells were seeded in a 24-well plate (24000/ well) in regular growth medium and incubated for 24h. Prior to lentiviral transduction, cells were washed and medium was changed to DMEM without FBS or antibiotics. Lentiviral particles were added to the cells at a MOI of 25. After 4h, regular growth medium was added to the cells. Transduced cells were selected with 1µg/ml puromycin (#A2856.0010 PanReac AppliChem) for 2 weeks. The following lentiviral particles were used: shANGPTL4_1: V3LHS_396197; shANGPTL4_2: V2LHS_97612; shNTC: V17081706.

Quantification of lipid species in ACMs

Free fatty acids (FFA), mono- (MG), di-(DG) and triacylglycerol (TG) were quantified by direct flow injection analysis (FIA) using Fourier Transform Mass Spectrometry (FIA-FTMS) as

described (2).Lipids were analyzed in negative ion mode dissolved in methanol/chloroform = 5/1 (v/v) containing 0.005 % dimethylamine.

Analysis of total FA in ACMs

Total FA analysis was performed by gas chromatography coupled to mass spectrometry (GC-MS) of FA methyl esters (FAMEs) as described previously (3).

Analysis of de novo synthesis of FA 16:0

MDA-MB-231, HCC38 and E0771 cells were grown to a confluency of 70% in 6-well plates. Cells were treated with basal medium, ACM, BSA (fatty-acid free) or BSA-OA containing 100µM $^{13}C_2$-acetate (#282014 Sigma-Aldrich). After 24 h, cells were washed twice with DPBS and lysates were harvested in 0.1% SDS. Enrichment of $^{13}C_2$-acetate in FA16:0 was analysed by mass isotopomer distribution analysis (MIDA) (4) using single ion monitoring of molecular ions (M0-M10: *m/z* 270-280).

RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

Total RNA of MDA-MB-231, HCC38 and E0771 cells was prepared using the QIAGEN RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN #74106). 2 µg of total RNA was reverse transcribed for 10 min at 25 °C, 50 min 42 °C and 15 min 70 °C using Superscript II reverse transcriptase (Invitrogen #100004925) and random hexamer primers (Roche #11034731001). qPCR was performed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne #0824) and a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) with following conditions: 95 °C 10 min, 40 cycles 95 °C 15s, 59 °C 30s and 72 °C 45s. Relative gene expression was calculated as $2^{-\Delta\Delta ct}$ as described before (5), using the untreated control cells as reference and *GAPDH* as housekeeping gene. Gene expression of mouse *Creb3l3* was quantified using TaqMan Assay (Thermo Fisher Scientific #4331182) and TaqMan Gene Expression Master Mix (Thermo Fisher Scientific # 4369016). TaqMan Assay was performed according to manufacturer´s instructions. Each experiment was performed in

triplicates and repeated at least three times. Primer sequences used for human und murine cell lines are shown in tab. S1.

Microarray analysis

Microarray analysis was performed as previously described (1). RNA integrity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, California). cRNA labeling and hybridization to the Illumina HT-12 v4 expression bead chips was carried out at the DNA Technologies core unit of the Center of Clinical Research (IZKF), University Hospital Leipzig. All steps were performed according to the manufacturer's specifications. Microarrays were scanned with an iScan array scanner (Illumina) and raw array data were processed and background subtracted in Illumina GenomeStudio. Further analysis was performed using the Chipster open source platform (6). Expression values were quantile normalized and log2-transformed using the Bioconductor package 'lumi' implemented in Chipster (7). Gene expression data of the 50 most up- and downregulated annotated genes were illustrated as heat maps using Morpheus software (8). The microarray data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number [E-MTAB-9485].

Pathway analysis with Metascape

Pathway analysis was performed using Metascape (9). In this gene set analysis, all genes that were up- or downregulated by ≥1.5-fold in MDA-MB-231 cells co-cultured with murine adipose tissue compared to control were included. Up- and downregulated genes were analyzed separately. We searched for pathways as defined by KEGG, with a p-value cutoff at 0.01.

Ingenuity Pathway Analysis

The analysis of upstream regulators was performed using Ingenuity Pathway Analysis (IPA) (10) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-

analysis). For this analysis, genes that were found to be up- or down-regulated by at least 1.5-fold in MDA-MB-231 cells upon co-culture with murine adipose tissue, were included. In the upstream regulator analysis tool we filtered for “transcription regulators” and “ligand-dependent nuclear receptors”.

Oil Red O staining of lipid droplets in MDA-MB-231 cells

MDA-MB-231 cells were fixed with 4 % paraformaldehyde diluted with DPBS with $MgCl_2$ $CaCl_2$ (Gibco® Life Technologies #14040-091). Oil Red O staining was performed using 0.3 % Oil Red O staining solution dissolved in isopropanol as previously described (5). Lipid droplets were visualized using phase contrast microscopy with 20 x magnification (Zeiss Primovert).

AdipoRed staining and quantification of lipid droplets in MDA-MB-231 and HCC38 cells

AdipoRed staining was performed using AdipoRed™ assay reagent (Lonza #PT7009) according to the manufacturer´s instructions and as previously described (5). MDA-MB-231 cells were seeded in 24-well plates and treated with 0.1 mM BSA or BSA-OA for 24 h, if not indicated otherwise. For quantification of intracellular lipids, the fluorescence intensity of stained cells was measured at excitation 485nm / emission 535nm at 400 points/ well using a Clariostar plate reader (BMG Labtech). The mean fluorescence intensity per well was determined. Lipid droplets were visualized using fluorescence microscopy with 20 x magnification (Zeiss Observer 7 inverted microscope).

Incucyte proliferation and wound healing assay with E0771 cells

Proliferation of E0771 cells was analyzed using the IncuCyte S3 Live-Cell Imaging System (Essen Bioscience, Ann Arbor, MI, USA). Cells were seeded in 100 µl complete growth medium at a density of 3.000 per well (n = 5) in 96-well plates (Eppendorf #0030730119) and incubated for 48h. Medium was then changed to basal medium and ACM, respectively, and the plate was placed into the IncuCyte device. Images were acquired at 10x magnification (4

positions per well) in a 2h interval for 24h. Analysis of proliferation was performed with the IncuCyte software 2019B Rev2 (Essen Bioscience, Ann Arbor, MI, USA) using the phase area confluence tool. For analyzing migration capability of E0771 cells, they were seeded into 96-well ImageLock plates (Essen Bioscience #4379) at a density of 15.000 per well in 100 µl complete growth medium. After an incubation period of 48h at 37 °C and 5% CO_2, the IncuCyte WoundMaker (Essen Bioscience) was used to create uniform, cell-free zones ("wounds") in the cell monolayers. Cells were washed once with basal medium and then incubated with basal medium or ACM in the IncuCyte S3 for 24h. The plate was scanned every two hours (10x objective, 1 position per well) and cell migration (given as relative wound density) was analyzed using the IncuCyte Scratch Wound analysis software.

ELISA

The concentration of secreted human Angiopoietin-like 4 protein in the cell culture supernatants was detected using a DY3485 Duo Set Human Angptl4 ELISA (R&D Systems # DY3485) as recommended by the manufacturer. Human transforming growth factor β (TGFβ) was quantified in the ACMs using human TGF-ß1 DuoSet ELISA (R&D Systems # DY240-05) according to manufacturer´s instructions using a Multiskan Go (Thermo Fisher) spectrophotometer.

Determination of FFA

FFA concentration of ACMs was determined using the NEFA-HR(2) Assay (Wako) according to the manufacturer´s protocol and as previously described (5). In brief, 150 µl of R1 reagent (#434-91795) was added to 3.5 µl of standard or ACM sample. After 2.5 min of incubation at 37 °C, the absorption was measured at 564 nm in a plate reader (Multiskan Go, Thermo Scientific) to determine the sample background. R2 reagent (#436-91995) was then added and the samples were further incubated for 4.5 min at 37 °C followed by a second measurement of absorption at 564 nm. The blank corrected absorbance vs concentration of the standards (#270-77000) was plotted to build a calibration curve (linear regression) and to determine the NEFA concentrations of the ACMs.

Western blot

Western blotting was performed as previously described (1). Whole cell lysates of MDA-MB-231, HCC38 and E0771 were prepared using RIPA Buffer supplemented with Proteinase Inhibitor Cocktail (Thermo Fisher Scientific # A32955) and PhosSTOP (Merck #4906837001). Cell lysates were separated by SDS-PAGE (NuPAGE 4-12% Bis-Tris Protein Gels, Thermo Fisher Scientific #NP0336) and transferred to PVDF membranes (Millipore #IPVH00010). For immunoblot analyses antibodies against Gapdh (Fitzgerald #10R-G109a), FAK (Cell Signaling #13009), pFAK(Tyr397) (Cell Signaling #8556), pFAK(Tyr576/77) (Cell Signaling #3281) and SREBP1 (Santa Cruz Biotechnology #sc-13551) were used as primary antibodies.

Gelatin zymography

MDA-MB-231 cells were washed with serum-free DMEM and incubated for 6 h with serum-free DMEM. Cell culture supernatants were collected and concentrated 45-fold using Amicon Ultra-2 centrifugal filters with a 10 kDa cut-off (Merck # UFC201024). The concentrates were mixed with non-reducing sample buffer (4 % SDS, 20 % glycerol, 0.01 % bromophenol blue, 125 mM Tris, pH 6.8) and transferred to a 12.5 % polyacrylamide gel containing 0.1 % SDS and 1 mg/ ml gelatin for PAGE. The volume of the cell culture supernatants was based on the protein concentration of whole cell lysates. The gel was then washed twice for 30 min with washing buffer (2.5 % Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$) to remove SDS followed by an incubation with incubation buffer (1 % Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$ and 1 µM $ZnCl_2$) for 24 h at 37 °C (gelatinase reaction). Staining was performed by incubation of the gel with staining solution (40 % methanol, 10 % acetic acid and 0.5 % Coomassie blue) for 1 h. The gel was then cleared with destaining solution (40 % methanol, 10 % acetic acid) until well-defined bands were detectable.

Supplementary Tables

Table S1

	forward	**reverse**
***ANGPTL4* (human)**	GGGAGAGGCAGAGTGGACTATTT	TTACTGTCCAGCCTCCATCTGA
***CREB3L3* (human)**	ATCGATGGCCTGGAGACTCG	TCCAAGAGGGACAGGTTTTGC
***PDK4* (human)**	GGAAGCATTGATCCTAACTGTGA	GGTGAGAAGGAACATACACGATG
***PLIN2* (human)**	TTGCAGTTGCCAATACCTATGC	CCAGTCACAGTAGTCGTCACA
***CPT1A* (human)**	GACTCTGGAAACGGCCAACT	AGGCCTCACCGACTGTAGAT
***SLC25A20* (human)**	TTGGGTTTGGTTTGGGGAAGA	GCTGCAAAAAGCTGGGGATAG
***FASN* (human)**	CTGTCTAGGTTTGATGCCTCCT	ACGATGGCTTCATAGGTGACTT
***SCD1* (human)**	TGCAGGACGATATCTCTAGC	ACGATGAGCTCCTGCTGTTA
***SREBF1* (human)**	CATCGACTACATTCGCTTTCTG	CCAGATCCTTCAGAGATTTGCT
***GAPDH* (human)**	GGCCTCCAAGGAGTAAGACC	AGGGGAGATTCAGTGTGGTG
***Angptl4* (mouse)**	CTCCGTGGGGACCTTAACTG	TTTTTACGCTCCTGCCGTTG
***Plin2* (mouse)**	GTCCCTCAGCTCTCCTGTTAG	ACATAAGCGGAGGACACAAGG
***Cpt1a* (mouse)**	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
***Slc25A20* (mouse)**	TATGTTCGCCGTGTGCTTCT	ATCCGTTCTCCAGGGGTCAT
***Acaa2* (mouse)**	AAAGACGGGACAGTCACAGC	CCAGGGGCGTGAAGTTATGT
***Fasn* (mouse)**	TGAATGGCATTACTCGGTCCCTGT	ATGCTCCAGGGATAACAGCACCTT
***Scd1* (mouse)**	TCCCTCCGGAAATGAACGAGAGAA	AGTGCAGCAGGACCATGAGAATGA
***Gapdh* (mouse)**	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT

Tab. S1: Primersequences used for human and murine cell lines.

Table S2

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
CREBBP	-1.138	transcription regulator	Activated	2.106	1.97E-03	ADAM12,AKAP12,CCR5,CD160,DUSP1,EGR1, EYA2,GH1,IFNA4,IFNB1,IRF6,MST1,NR4A3, PKHD1,PKLR,SFTPA1,TAGLN,TIPARP
PPARD	-1.137	ligand-dependent nuclear receptor	Activated	2.123	4.16E-03	ACAA2,ACADVL,ANGPTL4,CPT1A,PCSK6,PDK4, PDPK1,PKLR,PLIN2,SLC25A20
PPARG	1.208	ligand-dependent nuclear receptor	Activated	2.631	5.46E-03	ACAA2,ANGPTL4,CAT,CDKN2C,CPT1A,EDN2, EGFR,GSTA1,IFNB1,IL1B,KLK11, LOC102724788/PRODH,MTTP,PC,PDK4,PLIN2, SLC25A20,TSC22D3
PPARA	-1.147	ligand-dependent nuclear receptor	Activated	3.100	1.16E-02	ACAA2,ACADVL,ANGPTL4,CA9,CAT,CDKN2C,CFH, CPT1A,MTTP,PC,PDK4,PKLR,PLIN2,PRDM16, SLC25A20,SYCP3
SIM1	-1.186	transcription regulator	Activated	3.148	1.19E-02	ANG,ANGPTL4,EGFR,EGR1,IFNA4,NFATC2,NMBR, POU3F2,TLX2,TSC22D3
SPIB	1.021	transcription regulator	Activated	2.144	1.68E-02	CCR5,EGR1,IFNA4,IFNB1,SLC22A12
ARNT2	-1.007	transcription regulator	Activated	3.000	2.60E-02	ANG,ANGPTL4,EGFR,EGR1,IFNA4,NFATC2,NMBR, TLX2,TSC22D3
MYOCD	1.280	transcription regulator	Activated	2.164	2.64E-02	CACNA1C,ITGB1BP2,NFATC2,SCN5A,TAGLN
EP300	-1.174	transcription regulator	Activated	2.423	3.50E-02	ADAM12,AKAP12,CD160,CPT1A,DUSP1,EGR1, EYA2,GCG,IFNA4,IFNB1,IRF6,PDK4,PKLR,TIPARP, TRHR
IRF8	1.005	transcription regulator	Activated	2.221	4.16E-02	EGR1,IFNA4,IFNB1,IL1B,MS4A1,PML
TP53	-1.048	transcription regulator	Activated	2.252	4.64E-02	A2M,ACAA2,ACADVL,ADGRB1,AKAP12, APOBEC2,CA9,CAT,CKMT1A/CKMT1B,DUSP1,EGF R,EGR1,F3,FAT2,FOXO3,FUT1,GDA,H19,IL1A, IL1B,IL21R,IL4I1,KANK3,LBR, LOC102724788/PRODH,MPZL2,MST1,NANOG,PC BP4,PDGFRA,PML,RAD17,RGS12,RLIM,RNASE4, RRM2B,TACC2,TNFRSF1B,TSC22D3,ZEB2
SMAD3	1.119	transcription regulator	Activated	2.307	4.68E-02	EGR1,ESR2,FOXO3,IFNB1,IL1B,NANOG,TAGLN, TPM3,ZEB2
ESR1	1.038	ligand-dependent nuclear receptor	Activated	2.523	1.00E00	CLDN1,COG5,CYP17A1,EGFR,EGR1,EML1,ESR2, H19,ICA1,IL1A,IL1B,KISS1,LEPR,MAP7,MAPT, MST1,NFE2L3,OTUB2,OVOS2,PDK4,PLIN2, PLXDC1,SLC6A4,SLCO1B1,SUFU,TSC22D3,VAV3, XK

Tab. S2: Upstream regulators of genes with increased expression in MDA-MB-231 cells co-cultured with murine adipose tissue (HFD). Table shows identified upstream regulators of differentially expressed genes in MDA-MB-231 cells co-cultured with adipose tissue of obese mice vs control. Analysis was performed using Ingenuity Pathway Analysis on genes with at least 1.5-fold expression difference. Results were filtered for predicted activation state “Activated”.

Table S3

Upstream Regulator	Exp Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
SREBF2	-1.095	transcription regulator	Inhibited	-3.207	5.07E-08	DHCR7,FADS2,FASN,FDFT1,HMGCS1,INSIG1,LDLR, MSMO1,PCSK9,SCD,SREBF1,THRSP
SREBF1	-1.786	transcription regulator	Inhibited	-3.790	1.33E-06	CSAD,DHCR7,ELOVL7,FADS2,FASN,FDFT1,HMGCS1, INSIG1,LDLR,LPIN1,MSMO1,NR0B2,PCK2,PCSK9, SCD,SREBF1,THRSP
NR1H3	1.058	ligand-dependent nuclear receptor	Inhibited	-2.708	5.86E-06	ABCG1,ACSL3,CD5L,FASN,FDFT1,LDLR,MYLIP, NR0B2,SCD,SREBF1,TRH
ESRRA	1.216	ligand-dependent nuclear receptor	Inhibited	-2.132	1.68E-04	CSAD,CYP19A1,HK1,HMGCS1,MSX1,NR0B2,PCK2, PPM1B,PPP2R2B
HNF1B	-1.126	transcription regulator	Inhibited	-2.121	1.58E-03	F11R,ID3,ID4,ITGB6,NID2,OCLN,PCSK9,PTF1A,PTGIS
PPARGC1B	-1.020	transcription regulator	Inhibited	-2.173	4.53E-03	ACADL,DHCR24,FASN,FDFT1,SCD
PLAG1	1.140	transcription regulator	Inhibited	-2.169	9.67E-03	IGF2,LSP1,NFATC1,SRPK3,WT1-AS
SOX4	1.006	transcription regulator	Inhibited	-2.360	1.33E-02	ARPP21,DBNDD2,IGF2,KANK1,KLK8,RAG1,SLC17A8, ST6GAL1,TNFRSF19
OTX2	1.164	transcription regulator	Inhibited	-2.207	1.57E-02	CPXM2,PAX2,PAX6,RHO,TYR
TAF7L	1.004	transcription regulator	Inhibited	-2.000	1.72E-02	GSG1,HK1,LPIN1,PRM2
PPARGC1A	1.347	transcription regulator	Inhibited	-2.091	2.01E-02	ACADL,CKMT2,FASN,INSIG1,LDLR,LPIN1,PCK2,SCD, SREBF1,TYR
FOXO1	1.002	transcription regulator	Inhibited	-2.164	2.07E-02	CKMT2,CLDN5,DEPDC1,EBF1,FASN,FGF18, IGLL1/IGLL5,ITGB6,MRPL57,NFATC1,PCK2,RAG1, SCD,SREBF1
SP1	-1.026	transcription regulator	Inhibited	-2.308	3.37E-02	CD28,COL1A2,CTH,CYP19A1,DHCR24,DMD,F7,FASN, FOLR2,GRIA1,ID4,IGF2,KCNQ2,LDLR,NGFR,PHOX2A, SREBF1,TRH,UBC
MITF	1.075	transcription regulator	Inhibited	-2.941	4.39E-02	C8orf44-SGK3/SGK3,CA14,CMA1,HPS4,KCNN2,MGAT4B, NGFR,PAX6,PSMC3IP,TYR
TP53	-1.048	transcription regulator	Inhibited	-2.596	4.81E-02	ABCC2,ACSL3,ATG4C,BAG1,BICD2,CCNB1IP1,CNN2, COL1A2,CSF1,CYP19A1,DHCR24,DHCR7,DLG1,F11R, FASN,FDFT1,FXN,FXYD3,GPR87,HIST2H4B,HMGCS1, ID3,IFFO1,IGF2,LPIN1,LSP1,MATN4,NCOR2,NID2, NR0B2,PCBP4,PEG3,PIDD1,PPM1B,PRKAG2,SCMH1, SREBF1,TNFRSF18,UBC,WNT10A,WNT2
TCF3	1.095	transcription regulator	Inhibited	-2.169	1.11E-01	EBF1,GPLD1,GRIA1,IGLL1/IGLL5,MSMO1,NR0B2, RAG1,ZP1
HSF1	-1.171	transcription regulator	Inhibited	-2.401	1.88E-01	AURKC,FASN,HSPB2,LDLR,NCOR2,UBC
RARA	-1.223	ligand-dependent nuclear receptor	Inhibited	-2.377	2.74E-01	ABCC2,CYP19A1,EPO,FOLR2,HOXA3,IFIT1B,TRH,XK
STAT5B	-1.064	transcription regulator	Inhibited	-2.236	3.03E-01	CKMT2,EBF1,SCD,TCAP,THRSP

Tab. S3: Upstream regulators of genes with decreased expression in MDA-MB-231 cells co-cultured with murine adipose tissue (HFD). Table shows identified upstream regulators of differentially expressed genes in MDA-MB-231 cells co-cultured with adipose tissue of obese mice vs control. Analysis was performed using Ingenuity Pathway Analysis on genes with at least 1.5-fold expression difference. Results were filtered for predicted activation state “Inhibited”.

Table S4

FA	ACM<30 mean [nmol/ml]	SEM	ACM>40 mean [nmol/ml]	SEM	p-value
FA8:0	0.23	0.19	0.17	0.05	6.66E-01
FA10:0	0.68	0.17	0.75	0.08	6.90E-01
FA12:0	1.60	0.25	3.22	0.45	6.54E-02
FA14:0	6.11	1.73	14.07	1.68	2.11E-02
FA14:1-c9	0.70	0.05	1.77	0.24	3.01E-02
FA15:0	0.46	0.11	1.22	0.14	9.33E-03
FA16:0iso	0.10	0.03	0.18	0.02	5.14E-02
FA16:0	29.00	2.33	85.74	7.09	3.76E-04
FA16:1-c9	6.76	0.71	21.16	2.62	7.89E-03
FA17:0	0.23	0.04	0.62	0.06	1.94E-03
FA18:0	3.65	0.62	8.83	1.12	2.09E-02
FA18:1-c9 (n-9)	52.79	3.18	140.80	11.03	3.92E-04
FA18:1-c11 (n-7)	2.19	0.09	6.98	0.64	6.55E-04
FA18:2-c9,c12 (n-6)	10.85	0.96	28.16	2.02	1.83E-04
FA18:3-c6,c9,c12 (n-6)	0.09	0.01	0.14	0.01	3.21E-02
FA18:3-c9,c12,c15 (n-3)	1.09	0.09	2.62	0.19	2.90E-04
FA20:3-c8,c11,c14 (n-6)	0.27	0.07	0.32	0.02	2.58E-01
FA20:4-c5,c8,c11,c14 (n-6)	1.33	0.16	1.57	0.06	9.78E-02
FA20:3-c11,c14,c17 (n-3)	0.00	0.00	0.01	0.01	1.84E-01
FA20:4-c8,c11,c14,c17 (n-3)	0.03	0.01	0.05	0.01	3.75E-01
FA20:5-c5,c8,c11,c14,c17	0.09	0.01	0.07	0.01	5.04E-01
FA22:4-(7c,10c,13c,16c)	0.10	0.04	0.18	0.01	8.14E-03
FA22:5-(7c,10c,13c,16c,19c)	0.12	0.03	0.15	0.01	3.92E-01
FA22:6-c4,c7,c10,c13,c16,c19 (n-3)	0.11	0.01	0.13	0.02	6.36E-01

Tab. S4: Characterization of the free fatty acid profile of ACMs$_{<30}$ and ACMs$_{>40}$. Free fatty acids in ACMs were quantified using GC-MS. Values are displayed in nmol/ml.

Table S5A

Term	Description	LogP	Symbols
GO:0016125	**sterol metabolic process**	**-5.71**	CYP19A1,DHCR7,DHCR24,INSIG1,LDLR,MSMO1,SCD,SREBF1,ABCG1, PCSK9,PCK2,PLCB3,TYR,PER2,GALR2,PTGIS,LIPG,FADS2,ACOT7,ACSM1, NCOR2,GUCA1C,PSMA8,CLYBL
GO:0032409	regulation of transporter activity	-5.14	ATP2A1,CRHR1,DMD,FGF14,GRIA1,GALR2,SYNGR3,WWP2,WNK2, NETO1,PCSK9,ANO9,KCNN2,KCNQ2,PER2,CAPN10,SEMG1,KCNN3, SLC17A8
GO:0006639	acylglycerol metabolic process	-4.77	INSIG1,LDLR,PCK2,PNLIPRP2,SREBF1,DGKI,LIPG,PCSK9
GO:0042632	cholesterol homeostasis	-4.69	INSIG1,LDLR,LIPG,ABCG1,MYLIP,ACSM1,PCSK9,CRHR1,CYP19A1, MIR130B,PCDHGA3
GO:0031279	regulation of cyclase activity	-3.29	CRHR1,GALR2,GUCA1C,P2RY13

Table S5B (subterms of „sterol metabolic process“)

Term	Description	LogP	Symbols
GO:0006695	cholesterol biosynthetic process	-5.40	DHCR7,DHCR24,INSIG1,MSMO1,SCD,SREBF1,ABCG1
GO:0008203	cholesterol metabolic process	-5.18	DHCR7,DHCR24,INSIG1,LDLR,MSMO1,SCD,SREBF1,ABCG1,PCSK9
GO:0090181	regulation of cholesterol metabolic process	-3.67	DHCR7,LDLR,SCD,SREBF1,ABCG1
GO:0006631	fatty acid metabolic process	-3.25	INSIG1,PCK2,PTGIS,MSMO1,SCD,SREBF1,PER2,LIPG,FADS2,ACOT7, ACSM1
GO:0006633	fatty acid biosynthetic process	-3.22	INSIG1,PTGIS,SCD,LIPG,FADS2,ACOT7,ACSM1
GO:0045540	regulation of cholesterol biosynthetic process	-3.04	DHCR7,SCD,SREBF1,ABCG1
GO:0008610	lipid biosynthetic process	-2.87	CYP19A1,DHCR7,DHCR24,INSIG1,LDLR,PCK2,PTGIS,MSMO1,SCD, SREBF1,LIPG,FADS2,ABCG1,ACOT7,ACSM1
GO:0046890	regulation of lipid biosynthetic process	-2.11	DHCR7,INSIG1,LDLR,SCD,SREBF1,ABCG1

Tab. S5: Enriched GO terms (Biological Process) in genes with ≥1.5-fold downregulation in MDA-MB-231 cells co-cultured with adipose tissue of HFD mice vs control. Overrepresentation was tested using Metascape. The complete Homo Sapiens gene list was used as reference set to test for overrepresentation. (A) Top 5 list of enriched GO terms are presented. (B) List of subterms for GO term “sterol metabolic process” are shown.

Table S6A

Term	Description	LogP	Symbols
GO:0032352	positive regulation of hormone metabolic process	-5.12	ADM,EGR1,HPN,IL1B,MIR96,ADH1B,CPT1A,GALR1,ICA1,KISS1, PDGFRA,FAM3B,ACAA2,OSBPL1A,LEPR
GO:0051610	serotonin uptake	-4.63	GPM6B,SLC6A4,SLC18A1
GO:0061476	response to anticoagulant	-4.43	EGR1,REG1A,GPIHBP1
GO:0045834	**positive regulation of lipid metabolic process**	**-4.31**	ADM,CPT1A,IL1B,PDGFRA,NR4A3,DGKZ,VAV3,MIR96,EGR1,PDK4, MIR21,PLIN2,ANGPTL4,AADAT,ADCY2,CCR5,FOXO3,SLC6A4,TFPI, TNFRSF1B,CLDN1,ADH1B,CBFA2T3,EDN2,GSTA1,ACAA2,SLC27A4, AGXT2,OSBPL1A,LEPR
GO:0008285	negative regulation of cell proliferation	-4.28	ADM,CBFA2T3,CDKN2C,DUSP1,HPN,IL1A,IL1B,IRF6,KISS1,PML, REG1A,XCL1,SLC6A4,MAGED1,TSPAN32,CD300A,MIR21,MIR221, MIR96

Table S6B (subterms of „positive regulation of lipid metabolic process“)

Term	Description	LogP	Symbols
GO:0045923	positive regulation of fatty acid metabolic process	-3.50	CPT1A,IL1B,NR4A3,MIR96
GO:0019217	regulation of fatty acid metabolic process	-2.78	CPT1A,IL1B,PDK4,NR4A3,MIR96
GO:0046320	regulation of fatty acid oxidation	-2.63	CPT1A,PDK4,NR4A3
GO:0006631	fatty acid metabolic process	-2.12	CPT1A,EDN2,GSTA1,IL1B,PDK4,NR4A3,ACAA2,SLC27A4,MIR96

Tab. S6: Enriched GO terms (Biological Process) in genes with ≥1.5-fold upregulation in MDA-MB-231 cells co-cultured with adipose tissue of HFD mice vs control. Overrepresentation was tested using Metascape. The complete Homo Sapiens gene list was used as reference set to test for overrepresentation. (A) Top 5 list of enriched GO terms are presented. (B) List of subterms for GO term “positive regulation of lipid metabolic process” are shown.

Supplementary Figures

Figure S1

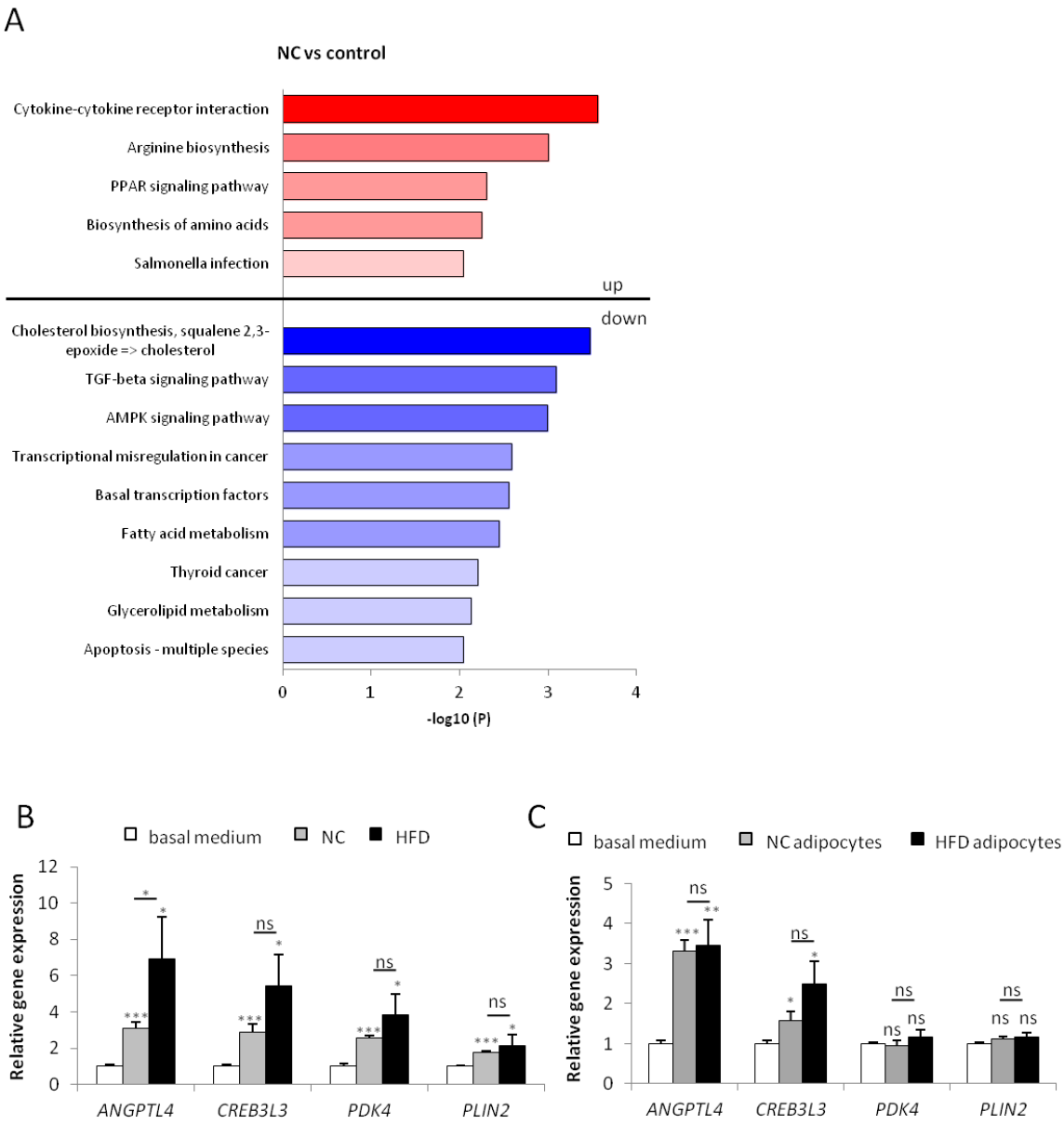

Fig. S1: Pathway Analysis and gene expression data of co-cultured MDA-MB-231 cells. (A) Over-represented cellular pathways (KEGG database) of ≥ 1.5-fold up- and downregulated genes in MDA-MB-231 cells co-cultured with adipose tissue of mice fed a normal chow diet (NC). (B) MDA-MB-231 cells were co-cultured with subcutaneous adipose tissue of NC or HFD mice. (C) Co-culture of MDA-MB-231 cells with isolated adipocytes of NC or HFD mice. (B+C) Gene expression data of MDA-MB-231 cells was determined by qPCR. Data are represented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise *p<0.05, **p<0.01, ***p<0.001, ns - not significant

Figure S2

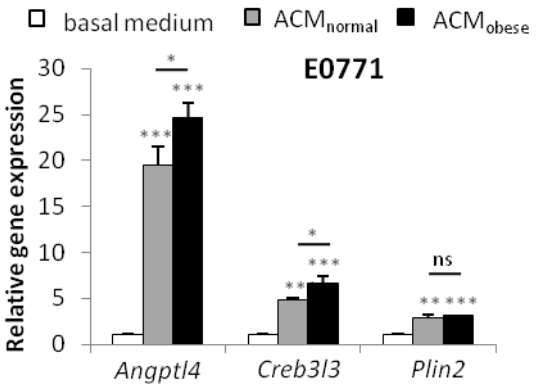

Fig. S2: ACM increases PPAR target gene expression in murine E0771 cells. E0771 cells were incubated with ACM of normal weight or obese mice. Data represent relative gene expression levels of PPAR target genes determined by qPCR. Data are presented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. *p<0.05, **p<0.01, ***p<0.001, ns - not significant

Figure S3

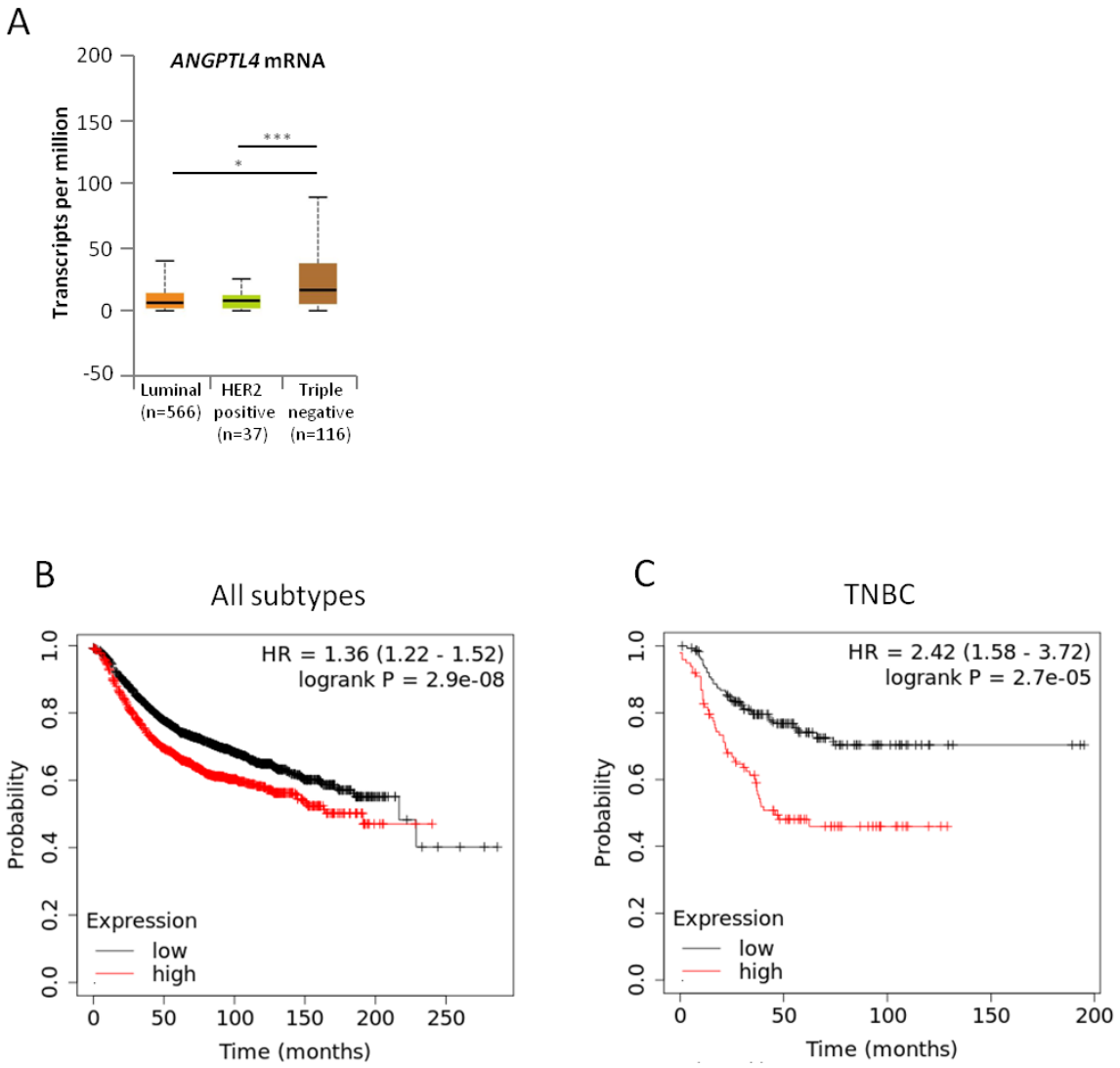

Fig. S3: ANGPTL4 mRNA is increased in human triple negative breast cancer and associated with reduced survival. (A) ANGPTL4 mRNA expression data in TCGA breast cancer samples according to molecular subtypes. Analysis was performed using the UALCAN portal (http://ualcan.path.uab.edu, Chandrashekar DS et al. Neoplasia 2017, Aug;19(8):649-658.). (B+C) Survival analysis in breast cancer patients for *ANGPTL4* mRNA expression and relapse-free survival (RFS). (B) Kaplan–Meier curves for all breast cancer subtypes and for (C) TNBC patients. Analysis was performed using the online tool Kmplot (www.kmplot.com; Mihaly Z et al. Breast Cancer Res Treat 2013). Hazard-ratios were calculated, at the best auto-selected cut-off and p-values were calculated using the logrank test.The most updated version of the database (July 2020) was utilized for the analyses.

Figure S4

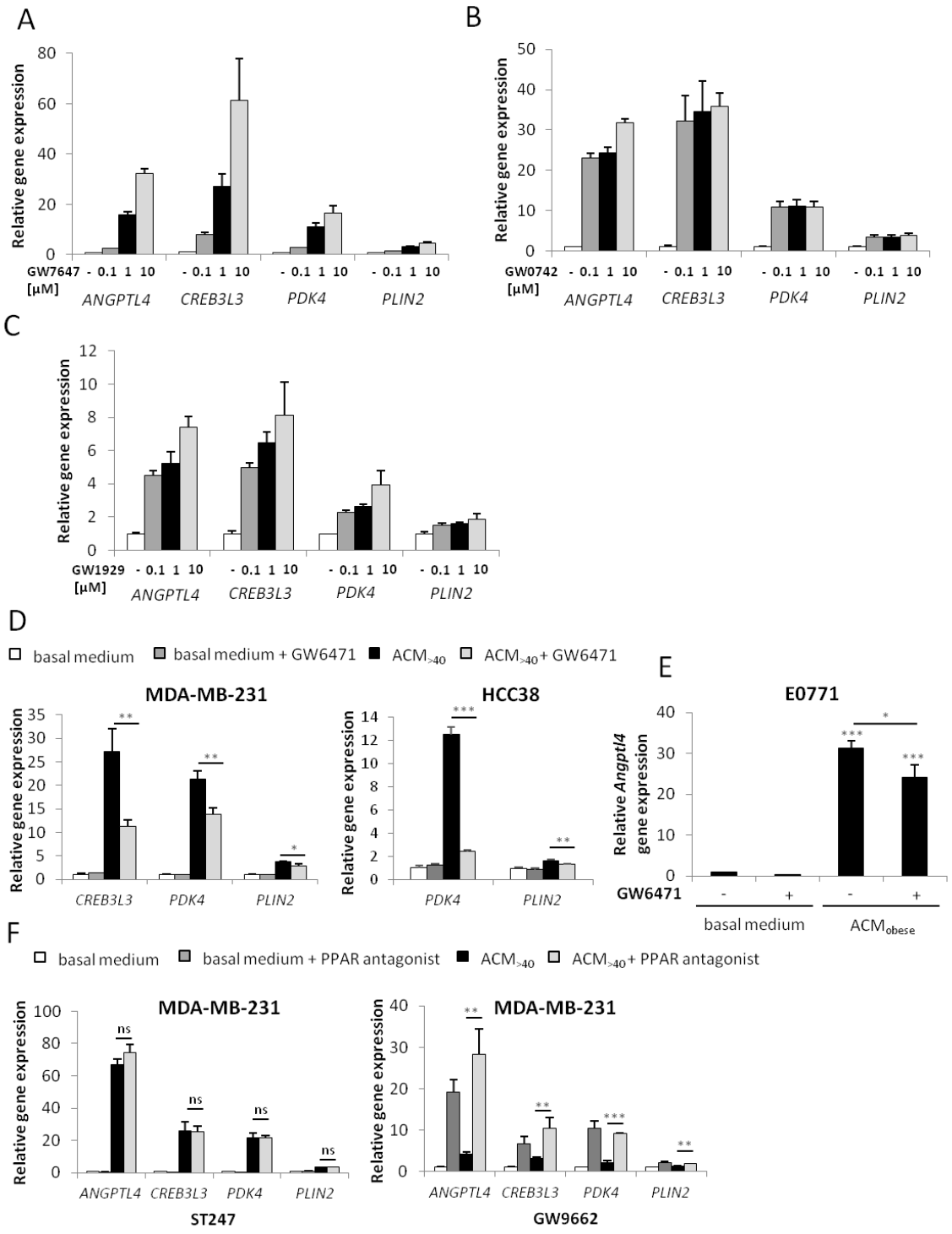

Fig. S4: PPAR target gene expression is dependent on PPARα. (A-C) MDA-MB-231 cells were treated with increasing concentrations of PPAR agonist of PPARα (GW7647), PPARβ (GW0742) or PPARγ (GW1929) for 24 h. (D+E) TNBC cells were incubated with ACM and treated with or without PPARα inhibitor GW6471. (F) Inhibitors of PPARβ (0.1 µM ST247) or PPARγ (10 µM GW9662) were added to MDA-MB-231 cells treated with basal medium or $ACM_{>40}$. (A-F) Data represent relative gene expression levels of PPAR target genes determined by qPCR. Data are presented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. *p<0.05, **p<0.01, ***p<0.001, ns - not significant

Figure S5

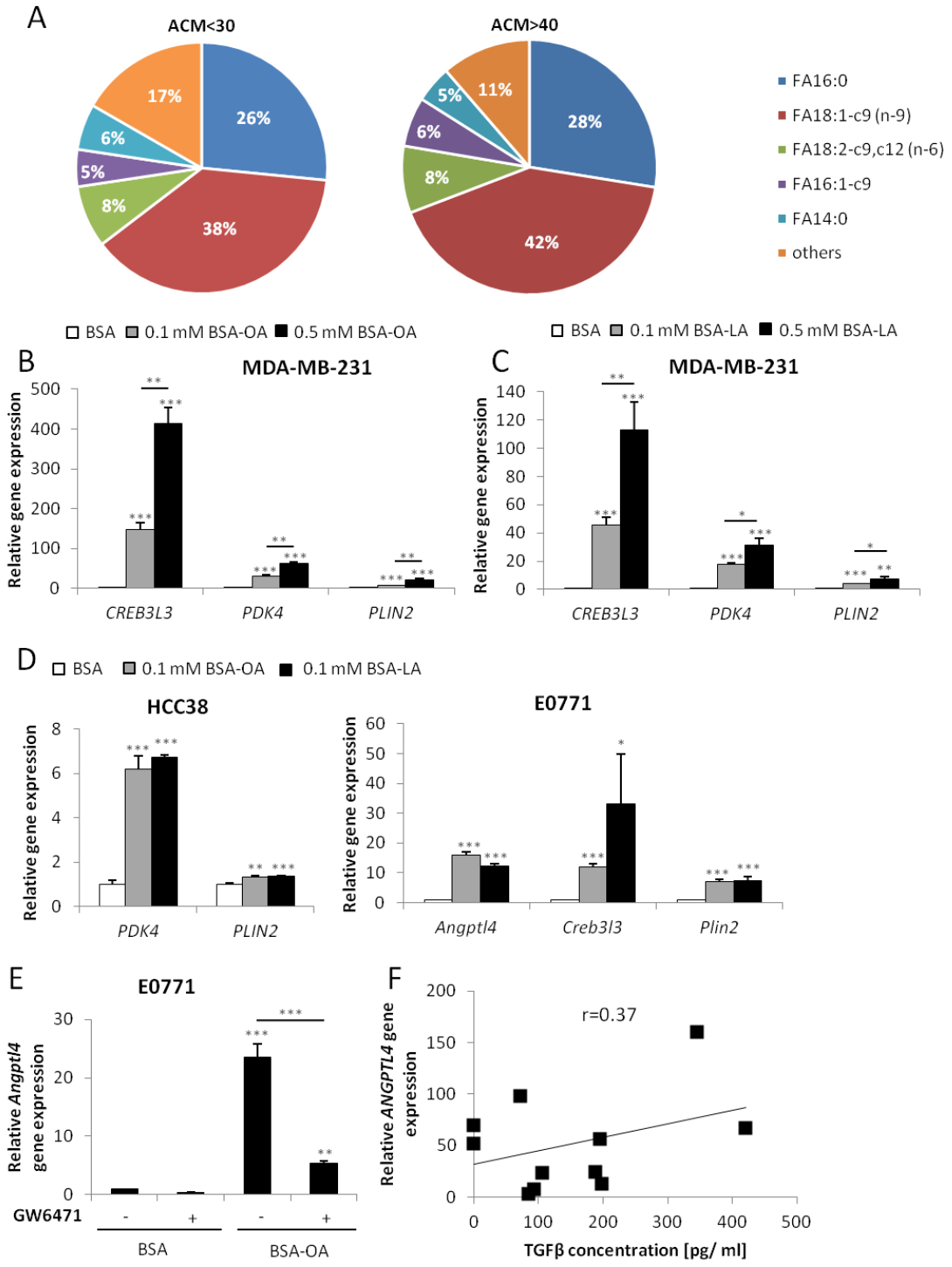

Fig. S5: Free fatty acids activate PPAR signaling in TNBC cells. (A) Pie charts show the relative fractions related to the sum of fatty acids in $ACM_{<30}$ and $ACM_{>40}$ as determined by GC-MS. (B-D) Gene expression data of PPAR targets in TNBC cells treated with BSA-OA or BSA-LA. (E) E0771 cells were incubated with BSA or BSA-OA with or without the PPARα inhibitor GW6471. (B-E) Relative gene expression levels were quantified by qPCR. Data are presented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. *p<0.05, **p<0.01, ***p<0.001. (F) Correlation analysis of the TGFβ concentrations in ACMs and *ANGPTL4* gene expression levels of MDA-MB-231 cells treated with corresponding ACMs.

Figure S6

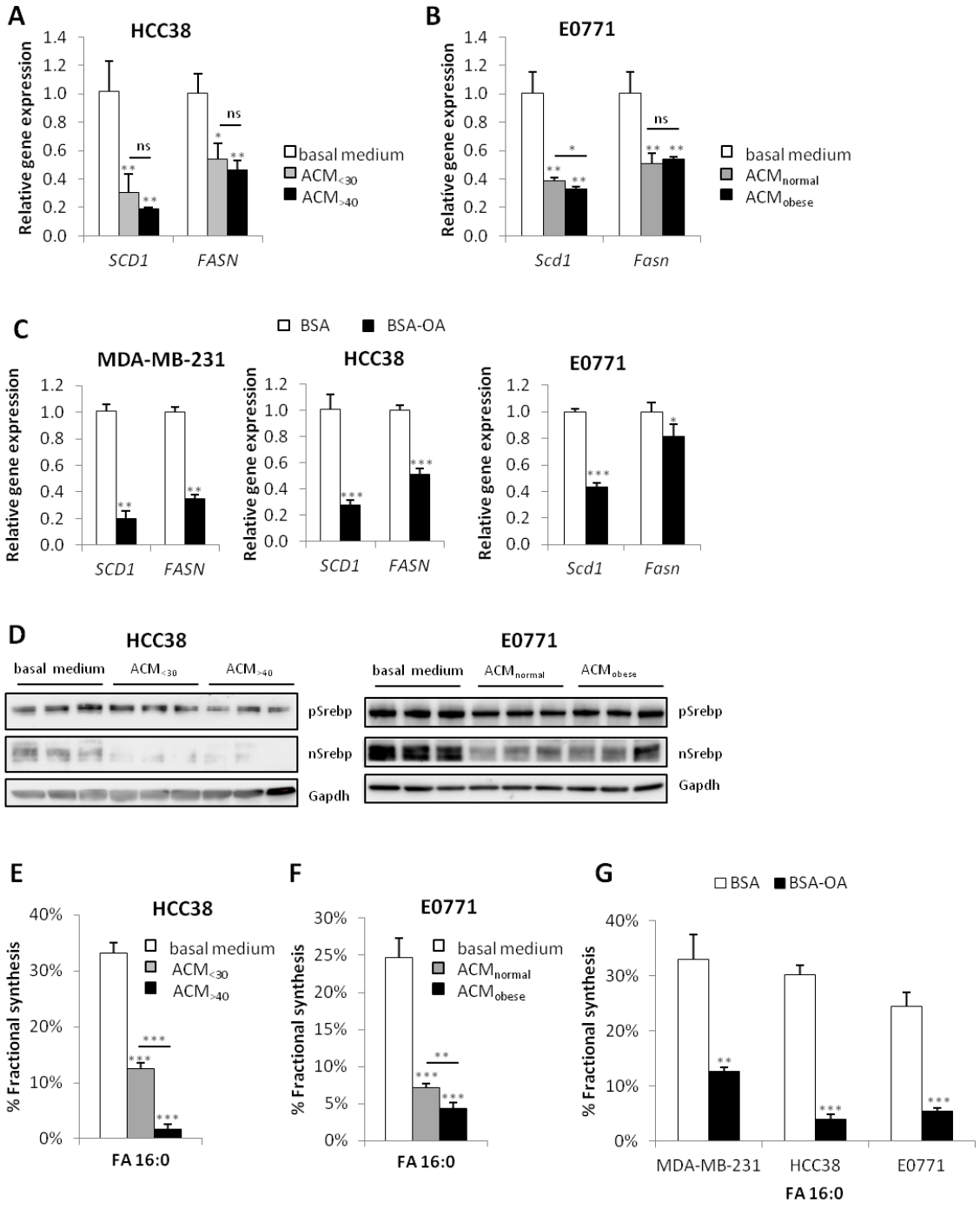

Fig. S6: Incubation of TNBC cells with ACM or BSA-OA decreases *de novo* FA synthesis. (A-C) Gene expression levels of *SCD1* and *FASN* in MDA-MB-231, HCC38 and E0771 treated with basal medium, ACM, BSA or BSA-OA were assessed by qPCR. (D) Immunoblots for Srebp1 precursor (pSrebp) and nuclear Srebp1 (nSrebp) in cell lysates of HCC38 and E0771 cells (E-G) Fraction of de novo synthesized palmitic acid (FA 16:0), calculated by MIDA, in HCC38, E0771 and MDA-MB-231 cells cultivated with ACM or BSA-OA. Data are presented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. *p<0.05, **p<0.01, ***p<0.001, ns – not significant

Figure S7

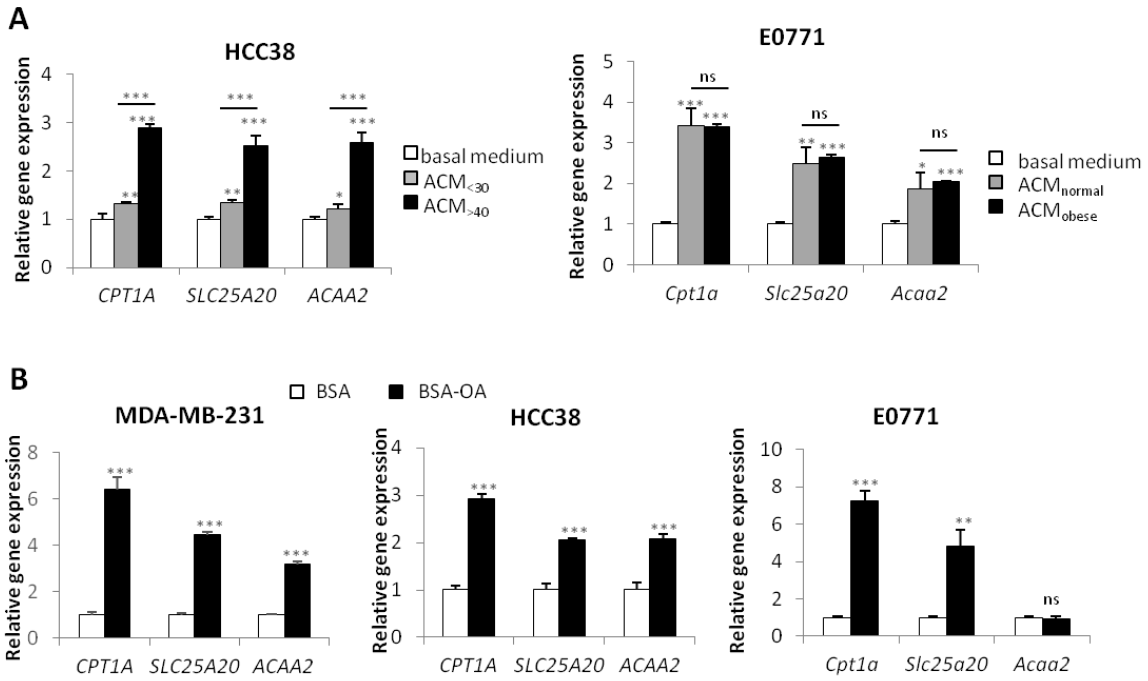

Fig. S7: Cultivation of TNBC cells with ACM or BSA-OA induces the expression of β-oxidation genes. (A) Relative gene expression levels of *CPT1A*, *SLC25A20* and *ACAA2* in TNBC cells treated with ACM or (B) BSA-OA was determined by qPCR. Data are presented as the means+SD of triplicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. *p<0.05, **p<0.01, ***p<0.001, ns – not significant

Figure S8

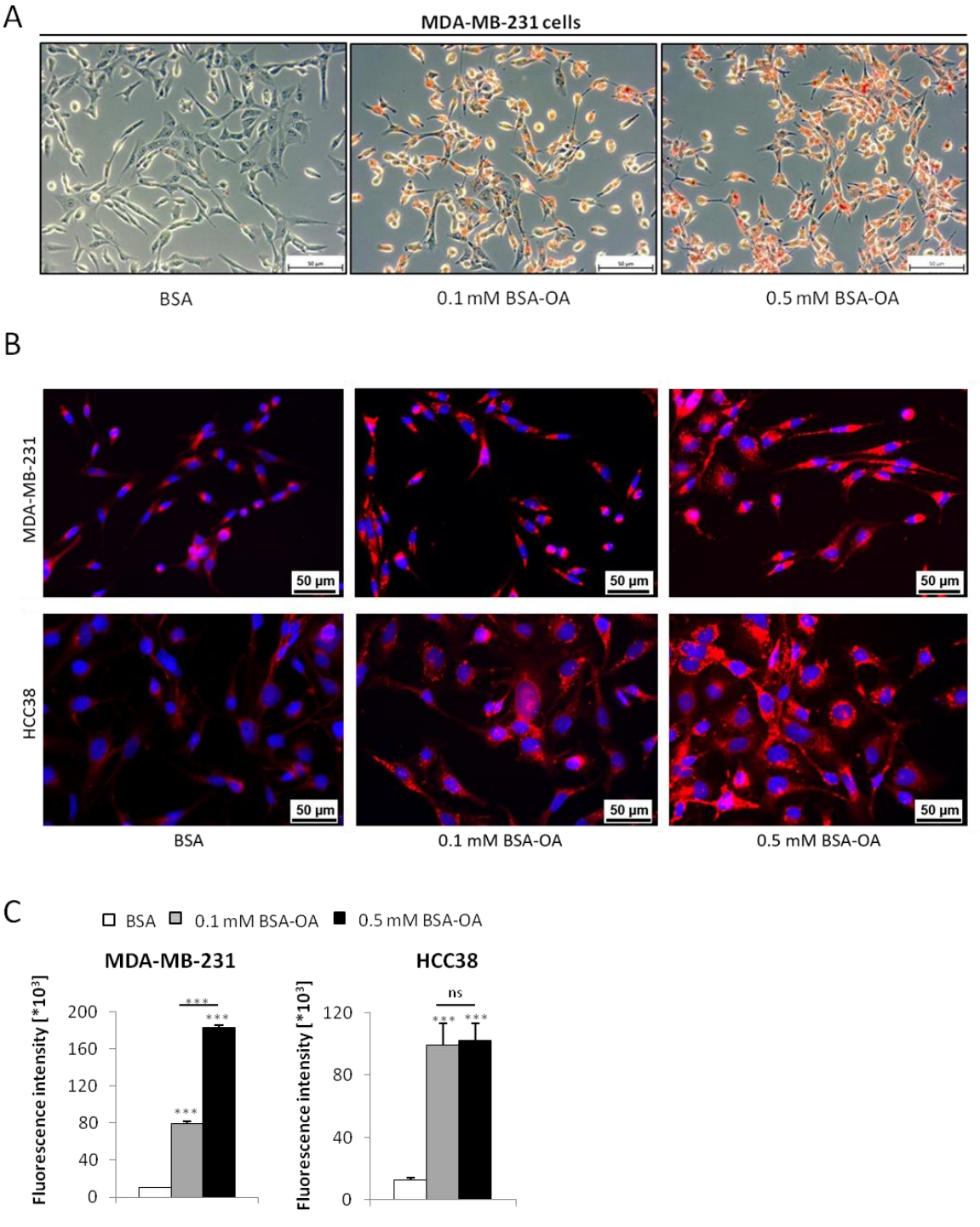

Fig. S8: Treatment with oleic acid increases intracellular lipid droplet formation in TNBC cells. (A) Oil Red O staining of MDA-MB-231 cells treated with BSA or BSA-OA. (B) AdipoRed staining of MDA-MB-231 and HCC38 cells cultivated with BSA or BSA-OA. (C) Quantification of AdipoRed staining. Data are presented as the means+SD of n=4 replicates of one representative experiment. Significance is relative to basal medium unless indicated otherwise. ***p<0.001, ns – not significant

Figure S9

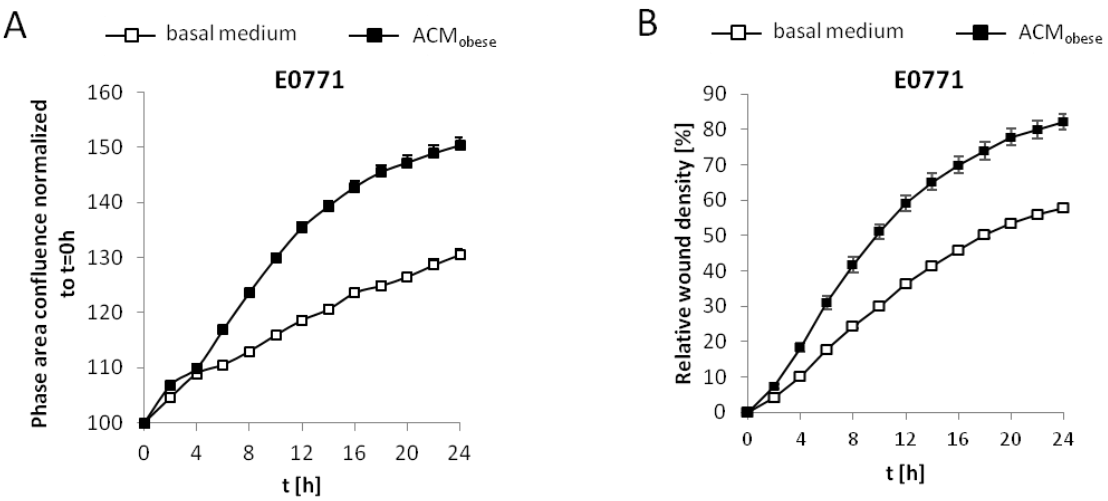

Fig S9: ACM promotes proliferation and migration of E0771 cells. (A) Proliferation of E0771 cells was assessed using the IncuCyte S3 Live-Cell Imaging system. Cells were treated with basal medium or ACM for 24h and confluence was scanned every 2h (10x objective, 4 positions per well). Data are presented as the mean of phase confluence area normalized to t = 0h. (B) Migration of E0771 was analyzed using the IncuCyte scratch wound assay. Wound closure was determined after treatment with basal medium or ACM after 24 h. Image acquisition was performed in a 2h interval (10x objective, one position per well) and data are presented as means of relative wound density. Data are represented as the means+SEM of n=5 replicates of one representative experiment.

Figure S10

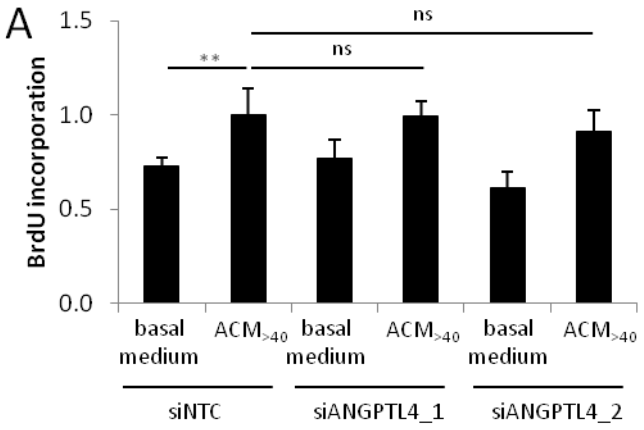

Fig. S10: *ANGPTL4* knockdown in MDA-MB-231 cells does not affect cell proliferation. (A) Cell proliferation of MDA-MB-231 cells was assessed after siRNA mediated knockdown of *ANGPTL4* (siANGPTL4_1 and siANGPTL4_2) by BrdU assay. Data are presented as the means+SD of n=6 replicates of one representative experiment. **p<0.01, ns - not significant

Figure S11

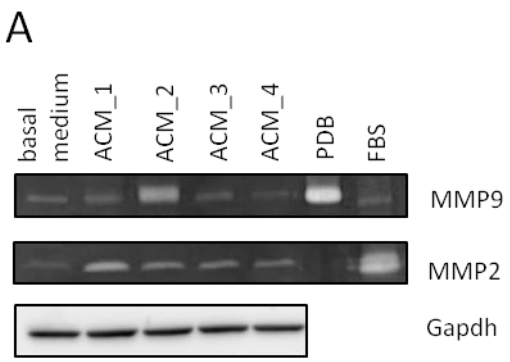

Fig. S11: MMP2 is upregulated in MDA-MB-231 cells upon ACM cultivation. MDA-MB-231 cells were incubated with basal medium or ACMs of different obese individuals (ACM_1-4). Cell culture supernatants were collected and the activity of MMP9 and MMP2 was analyzed using gelatin zymography. PDB - phorbol-12,13-dibutyrate. FBS – fetal bovine serum.

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