A serum-free and insulin-supplemented cell culture medium ensures fatty acid synthesis gene activation in cancer cells

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Abstract

While investigating the role played by *de novo* fatty acid biosynthesis (DNFA) in cancer cells, we sought a medium condition that would support cell proliferation without providing any serum lipids. Here we report that a defined serum free cell culture medium condition containing insulin, transferrin and selenium (ITS) supports controlled study of DNFA regulation in melanoma cell lines. This lipid-free ITS medium is able to support proliferation of melanoma cell lines that fulfill their lipid requirements via DNFA. We show that the ITS medium stimulates gene transcription in support of both DNFA and *de novo* cholesterol synthesis (DNCS), specifically mediated by SREBP1/2 in melanoma cells. We further found that the ITS medium promoted SREBP1 nuclear localization and occupancy on DNFA gene promoters. Our data show clear utility of this serum and lipid-free medium for melanoma cancer cell culture and lipid-related areas of investigation.

Introduction

de novo fatty acid biosynthesis (DNFA) is the metabolic pathway that converts carbohydrates into fatty acids. In healthy adults, DNFA occurs in liver and adipose tissues for energy storage or distribution to other tissues. Many malignant cancer cells also exhibit elevated DNFA as a hallmark adaptation to support proliferation and survival (1, 2). Certain cancer cells can proliferate entirely by relying on lipids generated from DNFA; others rely additionally upon lipid uptake (3). However, DNFA appears to be required for cell survival even with the presence of external lipids, suggesting that DNFA is essential for cancer cells regardless of proliferative state (3). Thus, DNFA is of particular interest as a potential therapeutic target for cancers (4, 5). DNFA is primarily regulated at the mRNA level of catalytic enzymes that drive the biosynthetic reactions (6), a transcriptional process under the control of the sterol regulatory element-binding protein 1 (SREBP1) (7). However, DNFA gene regulation is still not fully understood at the molecular level. Cell culture studies of DNFA with widely-accepted serum-containing medium conditions are often confounded by the presence of external lipids, with consequent difficulty to disentangle the respective effects of lipid synthesis and lipid update, both of which may occur even among cells that are able to survive and proliferate using DNFA alone (7, 8).

Most cell culture media are composed of serum supplements and basal mediums (BMs), each of which may contain confounding factors for DNFA studies. Typical serum often contains external lipids in two forms: non-esterified free fatty acids (FFAs) associated with albumin, and lipoproteins that carry triglycerides, cholesterol and phospholipids encapsulated by apoproteins (9, 10). Cells can take up FFAs *via* physical diffusion across the cellular membrane (11) or *via* active transport aided by membrane-associated protein CD36 or FATPs (12). Lipoprotein uptake occurs when a lipoprotein binds to a cell surface receptor (e.g. LDL receptor; LDLR) and the bound

lipoprotein/receptor complex undergoes endocytosis (13). After transport from serum to cells, both FFAs and lipoprotein lipids are potent DNFA inhibitors (14). Intracellular cholesterol interferes with retrograde trafficking of SREBP1 from the ER to the Golgi apparatus – an essential step in post-translational processing and maturation of SREBP1 – and consequently inhibits DNFA enzyme expression (7). Polyunsaturated FFAs have also been observed to inhibit both SREBP1 mRNA transcription and protein maturation, resulting in decreased expression of DNFA enzyme genes (15-17). Lipid metabolism studies have often employed lipoprotein-deficient serum (LPDS), in which lipoproteins are removed by ultracentrifugation, but FFAs – which are confounding for DNFA studies – are retained (18). An alternative is delipidated serum (8), but that is prepared by organic solvent extraction and is not completely lipid-free (19). Preparation protocols vary widely in organic solvent composition and extraction time, and quality variation between batches is common (20, 21).

Basal media (BM) often contain glucose, which besides its bioenergetic role as fuel for ATP synthesis also serves as a carbon source for biosynthesis of amino acids, nucleotides, other carbohydrates, and lipids. Two of the most common BMs for cancer cell culture are Roswell Park Memorial Institute (RPMI) 1640 medium (22), with 11.11 mM glucose concentration (normal glucose level); and Dulbecco's modified Eagle's medium (DMEM), which contains 25 mM glucose (high glucose level). In cultured hepatocytes and adipocytes, high glucose conditions stimulate lipogenesis and DNFA gene expression (23, 24). Healthy livers employ glucose for DNFA principally by glycolytic citrate generation and the tricarboxylic acid (TCA) cycle with oxidative phosphorylation (25, 26). Glycolysis produces pyruvate that mitochondria metabolize into citrate and ATP in the TCA cycle. The citrate then translocates to cytosol where it is cleaved by ATP-citrate lyase (ACLY) to produce acetyl-CoA as a substrate for DNFA (27-29). In contrast to healthy livers, tumors display enhanced glycolytic

activity but impaired oxidative phosphorylation. Pyruvate is often metabolized to lactate within the cytoplasm of cancer cells preferentially to the TCA cycle (Warburg effect) (30). Furthermore, elevated glucose stimulates glycolysis and inhibits aerobic respiration in tumors (Crabtree effect) (31). Thus, high-glucose BMs promote conversion of glucose to pyruvate and NADH generation but inhibit production of substrates for DNFA via the TCA cycle (32, 33). In general, the glucose content of DMEM may distort normal cellular behavior and thus render it unsuitable for molecular study of DNFA transcription regulation. Both aerobic respiration and anaerobic glycolysis contribute to glucose catabolism in cancer cells cultured with RPMI-1640 medium (34). Therefore, between the two common BMs, RPMI-1640 seems preferable for lipogenesis-related investigations of cancer cells.

The commercially available insulin, transferrin, and selenium (ITS) supplement is a serum replacement, supporting cell survival and growth but containing no lipids. ITS supplement has been used for *in vitro* culture of mesenchymal stem cells isolated from adipose or cartilage tissues, to maintain their differentiation and proliferation capacities for tissue transplantation (35, 36). Insulin is a growth factor with a mitogenic effect in cell culture that promotes the uptake of glucose and amino acids (37, 38). In livers, insulin stimulates *SREBF1c* mRNA expression (23) as well as its proteolytic processing to stimulate DNFA gene expression (23, 39). Transferrin is a glycoprotein that transports Fe^{3+} in blood plasma and delivers Fe^{3+} to cells through binding to transferrin receptor on cell surface (41). Fe^{3+} is an essential component of heme-containing enzymes like cytochromes for oxidative phosphorylation process and various non-heme iron enzymes, such as ribonucleotide reductase for DNA synthesis (42). Transferrin provides Fe^{3+} necessary to support cell survival and proliferation in culture (43). Selenium is required for proper function of antioxidant enzymes, including glutathione peroxidase and thioredoxin reductase, in which selenocysteine is

indispensable for their catalytic activities (44). Components of ITS have been individually assembled in various serum-free media to support cell growth in cancer studies (45), but reports relevant to lipid metabolism are missing. Here, we introduce the combination of RPMI-1640 and ITS (ITS medium) as a straightforward serum-free medium condition for activating DNFA gene expression in cancer cell culture. The ITS medium stimulates cell growth, exhibits consistent effect across batches, and is free of confounding lipid factors. We expect that it may be adopted for investigations in lipid metabolism and will facilitate *in vitro* screening for inhibitors of DNFA pathway.

Materials and Methods

Cell culture reagent

The melanoma cell line HT-144 was obtained from the MGH Center for Molecular Therapeutics. Cell line was cultured in RPMI-1640 medium (21870092, Thermo Fisher Scientific) with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and 50 U/ml penicillin-streptomycin (Gibco) in a 37 °C incubator with 5% CO_2. 0% FBS medium contained RPMI-1640 medium, with 2 mM L-glutamine (Gibco) and 50 U/ml penicillin-streptomycin (Gibco). 1% ITS medium contained the RPMI-1640 medium with 1× Insulin-Transferrin-Selenium (ITS-G, Thermo Fisher Scientific), 2 mM L-glutamine (Gibco) and 50 U/ml penicillin-streptomycin (Gibco).

Cell proliferation assay

For proliferation assay, HT-144 cells were seeded at a density of 5,000 cells per well in 24-well plates (Corning) in RPMI-1640 medium with10% FBS. Sixteen hours after seeding, cells were washed twice with PBS buffer and then cultured in three

different medium conditions for 6 days. Relative cell viability was quantified with the alamarBlue cell viability reagent (DAL1025, Thermo Fisher Scientific) and fluorescence signals were measured with the Envision 2103 multilabel microplate reader (Perkin Elmer). Each data point was measured with four replicates. Because the culture medium influences resazurin fluorescence, the background fluorescence of medium-only with alamarBlue reagent was subtracted for data normalization.

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase (Qiagen). RNA concentrations were quantified with Qubit™ RNA BR assay kit (Thermo Fisher Scientific). 1 μg RNA was used for cDNA synthesis with RNA to cDNA EcoDry™ premix (TaKaRa) containing both random hexamer and oligo(dT)18 primers (Double Primed). qPCR was carried out in triplicates on a LightCycler® 480 instrument (Roche) using LightCycler® 480 SYBR green I master (Roche). qPCR primers were pre-designed by MGH primer bank (https://pga.mgh.harvard.edu/primerbank/) and the primer sequences are listed in Table 1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to the 18S housekeeping gene, and the mean of negative control samples was set to 1.

Table 1. The primers used for RT-qPCR.

ACACA forward	5’ – ATGTCTGGCTTGCACCTAGTA – 3’
ACACA reverse	5’ – CCCCAAAGCGAGTAACAAATTCT – 3’
ACLY forward	5’ – TCGGCCAAGGCAATTTCAGAG – 3’
ACLY reverse	5’ – CGAGCATACTTGAACCGATTCT – 3’
ACSS2 forward	5’ – AAAGGAGCAACTACCAACATCTG – 3’

ACSS2 reverse	5' – GCTGAACTGACACACTTGGAC – 3'
FASN forward	5' – AAGGACCTGTCTAGGTTTGATGC – 3'
FASN reverse	5' – TGGCTTCATAGGTGACTTCCA – 3'
SCD forward	5' – TCTAGCTCCTATACCACCACCA – 3'
SCD reverse	5' – TCGTCTCCAACTTATCTCCTCC – 3'
ACSL1 forward	5' – CCATGAGCTGTTCCGGTATTT – 3'
ACSL1 reverse	5' – CCGAAGCCCATAAGCGTGTT – 3'
SREBF1 forward	5' – ACAGTGACTTCCCTGGCCTAT – 3'
SREBF1 reverse	5' – GCATGGACGGGTACATCTTCAA – 3'
SREBF2 forward	5' – AACGGTCATTCACCCAGGTC – 3'
SREBF2 reverse	5' – GGCTGAAGAATAGGAGTTGCC – 3'
HMGCS1 forward	5' – GATGTGGGAATTGTTGCCCTT – 3'
HMGCS1 reverse	5' – ATTGTCTCTGTTCCAACTTCCAG – 3'
HMGCR forward	5' – TGATTGACCTTTCCAGAGCAAG – 3'
HMGCR reverse	5' – CTAAAATTGCCATTCCACGAGC – 3'
LDLR forward	5' – ACCAACGAATGCTTGGACAAC – 3'
LDLR reverse	5' – ACAGGCACTCGTAGCCGAT – 3'
18S forward	5' – GTAACCCGTTGAACCCCATT – 3'
18S reverse	5' – CCATCCAATCGGTAGTAGCG – 3'

siRNA transfection

The human-specific siRNAs targeting *SREBF1* (6720) and *SREBF2* (6721) were the pre-designed ON-TARGETplus SMARTpool siRNA reagents from Dharmacon. Each ON-TARGETplus SMARTpool siRNA was a mixture of four siRNA duplexes. siRNAs were suspended in RNase-free 1 × siRNA Buffer (Dharmacon) to

yield 20 μM stock solutions. HT-144 cells were transfected with siRNAs at final concentration of 50 nM using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) at a density of 2×10^5 cells/ well in a 6-well plate. For each transfection, 2.5 μl of siRNA stock solution was mixed with 4 μl of Lipofectamine RNAiMAX in 200 μl of Opti-MEM I medium and then incubated for 10-20 minutes at room temperature. HT-144 cells were diluted in 10% FBS/RPMI-1640 medium without antibiotics so that 800 μl medium contains 2.5×10^5 HT-144 cells. 200 μl of siRNA/Lipofectamine RNAiMAX complexes was mixed with 800 μl of the diluted HT-144 cells in one well of a 6-well plate. Transfected HT-144 cells were incubated at 37°C for 16 hours before medium changes.

Immunoblot assay

HT-144 cells were seeded in 10 cm^2 plates (Corning) in 10% FBS/RPMI-1640 medium at day one. Sixteen hours after seeding, cells were washed twice with PBS buffer and then cultured in different medium conditions. Total cell lysate was harvested with RIPA buffer containing protease inhibitors (protease inhibitor cocktail tablets, Roche) on the third day. Nuclear and cytoplasmic protein fractions were prepared with NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). Protein samples were separated on the 4-15% Mini-PROTEAN® TGX™ precast SDS-PAGE gels (Bio-Rad) and then transferred to polyvinyl difluoride (PVDF) membranes (Immobilon-P, Millipore) for immunoblot analysis. The following primary antibodies were used: mouse anti-SREBP1 (IgG-2A4, BD Biosciences), rabbit anti-FASN (C20G5, Cell Signaling), rabbit anti-SCD (23393-1-AP, Proteintech), rabbit anti-ACSL1 (D2H5, Cell Signaling), rabbit anti-histone H3 (9715, Cell Signaling) and rabbit anti-beta-actin (13E5, Cell Signaling). After being incubated with primary antibodies overnight in PBST solution with 5% non-fat dry milk, immunoblot

membranes were probed with HRP-conjugated affinity-purified donkey anti-mouse or anti-rabbit IgG (GE Healthcare) as secondary antibodies and visualized with the immobilon Western Chemiluminescent HRP substrate (Millipore).

Chromatin immunoprecipitation (ChIP) quantitative PCR (ChIP-qPCR)

For each ChIP assay, 5×10^7 HT-144 cells were used. HT-144 cells were seeded in 10% FBS medium, and then medium was replaced with 10% FBS or 1% ITS medium and cultured for 24 hours before ChIP-qPCR analyses. Chromatin from HT-144 cells was fixed with 1% formaldehyde (Polysciences) and prepared with Magna ChIP™ HiSens chromatin immunoprecipitation kit (EMD Millipore). Nuclei were sonicated on a sonic dismembrator 550 (Fisher Scientific) with a microtip (model 419) from Misonix Inc. Lysates were sonicated on ice with 10 pulses of 20 seconds each (magnitude setting of 3.5) and a 40-sec rest interval. The supernatant was used for immunoprecipitation with the following antibodies: rabbit anti-SREBP1 (H-160, Santa Cruz Biotechnology), rabbit anti-CBP (C-22, Santa Cruz Biotechnology), rabbit anti-RNA polymerase II (8WG16, BioLegend) and rabbit anti-histone H3 (acetyl K27) (ab4729, Abcam). qPCR reactions in triplicates were performed on a LightCycler® 480 instrument (Roche) using LightCycler® 480 SYBR green I master (Roche). The ChIP-qPCR primers were designed with software Primer 3. The primer sequences are listed in Table 2.

Table 2. The primers used for ChIP-qPCR.

SCD 12F (TSS)	5' – GTGGCACCAAATTCCCTTCG – 3'
SCD 12R (TSS)	5' – GACACCGACACCACACCA – 3'
SCD 245F	5' – CTTGGCAGCGGATAAAAGGG – 3'
SCD 245R	5' – GCACGCTAGCTGGTTGTC – 3'

206

207 # Results

208 ## A lipid-free and insulin-supplemented (ITS) medium

209 ## supports melanoma cell proliferation

210 We performed time-course analysis to measure cell growth rates of a melanoma
211 cell line, HT-144, under three different cell culture medium conditions: RPMI-1640
212 supplemented with either 10% FBS, 0% FBS, or 1% ITS. Viable HT-144 cells were
213 quantified with alamarBlue daily for six days. We found that HT-144 cells cultured in
214 10% FBS and 1% ITS medium conditions displayed a time-dependent increase of
215 fluorescence reads in the alamarBlue assay (Fig. 1A). We also observed increased cell
216 number, which confirms that HT-144 cells proliferate in both 10% FBS and 1% ITS
217 medium conditions. Because 1% ITS medium does not contain any external lipids, the
218 lipids for membrane synthesis during proliferation are products entirely derived from
219 DNFA. We observed a growth plateau of HT-144 cells in 0% FBS medium, which lacks
220 growth factors such as insulin, and in which HT-144 cells therefore remain quiescent.
221 The alamarBlue assay measures the fluorescence emission of resorufin
222 molecules converted from resazurin by reductants such as NADPH or NADH (46, 47).
223 The alamarBlue assay is thus an indicator of cellular NADPH/NADH concentration in
224 living cells. Because cytosolic NADPH serves as the reductant for biosynthesis
225 pathways such as lipid and nucleic acid synthesis, alamarBlue assay may provide a hint

of the DNFA activity. Therefore, we compared the alamarBlue reads of cells cultured in different medium conditions at day one, when they had the same cell numbers in the three medium conditions. HT-144 cells cultured in 1% ITS medium had the highest fluorescence reads, whereas those in 10% FBS medium had the lowest reads (Fig. 1B). This result suggests that even when the cell number remains the same, HT-144 cells cultured in 1% ITS possibly have higher NADPH/NADH concentrations.

Fig 1. A lipid-free and insulin-supplemented medium supports proliferative cell state in melanoma cells.

(A) HT-144 cells were seeded in 10% FBS medium at day zero. On day one, cells were washed with PBS and changed to the indicated medium conditions. Cell proliferation was measured with alamarBlue assay at each day. Each data point represents the mean ±SD of quadruplicate samples. The results were analyzed using two-way repeated measures ANOVA followed by *post hoc* Tukey's multiple comparison tests. For culture time, $F = 1505$, $P < 0.0001$; for culture condition, $F = 2153$, $P < 0.0001$; for interaction between culture time and condition, $F = 409.4$, $P < 0.0001$. (B) HT-144 cells were seeded in 10% FBS medium at day zero. On day one, cells were washed with PBS and changed to the indicated medium conditions. alamarBlue assay was performed on the cells cultured in the indicated medium for one hour. Results were analyzed using one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests. $F = 202.7$. Significant differences between medium conditions are indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$. ns, not significant.

ITS medium increases DNFA and DNCS gene expression in melanoma cells

To determine whether the increase of fluorescence emission by resorufin in 1% ITS condition (Fig. 1B) was due to elevated DNFA activity, we performed RT-qPCR analyses of DNFA mRNA, including *ACLY*, *ACSS2*, *ACACA*, *FASN*, *SCD*, and *ACSL1*. We also examined the expression of *de novo* cholesterol synthesis (DNCS) genes, including *HMGCS1* and *HMGCR*. HT-144 cells were seeded in 10% FBS medium, and then medium was replaced with 0% FBS or 1% ITS medium and cultured for 24 hours before RT-qPCR analyses. We observed a significant increase in the expression of DNFA (Fig. 2A-F), DNCS (Fig. 2G, H) and *LDLR* (Fig. 2I) genes when comparing cells cultured in 0% FBS and 1% ITS medium conditions to cells in 10% FBS medium. DNFA and DNCS gene expression remained higher in 0% FBS and 1% ITS three days after changing the media (data not shown). These results suggest that the increase in lipogenic gene expression persists in cells cultured in 0% FBS and 1% ITS media. We observed lower DNFA and DNCS gene expression in cells cultured in 10% FBS medium than in 0% FBS medium, consistent with repression of DNFA and DNCS by lipids derived from the serum supplement.

Figure 1A indicates that HT-144 cells remain quiescent in 0% FBS medium and proliferate in 1% ITS. Quiescent HT-144 cells have enhanced DNFA and DNCS gene expression, even though there is no requirement for membrane lipid synthesis to support proliferation, and the reasons for this remain unclear. DNFA and DNCS gene expression is elevated in 1% ITS medium as compared with 0% FBS medium, which may suggest that the insulin component of the ITS supplement contributes to stimulation of DNFA gene expression.

We further observed significantly increased expression of *SREBF1* and *SREBF2* genes in cells cultured in 0% FBS and 1% ITS medium conditions, compared

to 10% FBS medium (Fig. 2J, K). These results suggest that SREBP1 and SREBP2 are transcriptionally activated in lipid-free and insulin-supplemented conditions. Because SREBP1/2 can bind to the sterol regulatory element (SRE) at its own promoter, SREBP1/2 protein possibly regulate its mRNA production through auto-activation (48) and potentially contributes to elevated DNFA and DNCS gene expression.

Figure 2. Lipid depletion combined with insulin supplement yields increased expression of lipogenic genes.

(A-K) The expression level of DNFA and DNCS genes was analyzed by RT-qPCR assay. HT-144 cells were cultured in 10%, 0% FBS or 1% ITS medium for 24 hours. Significant differences between medium conditions are indicated as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 using one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests. ns, not significant. Each data point represents the mean ±SD of results from quadruplicate samples.

ITS medium increases DNFA and DNCS gene expression via SREBP1 and SREBP2, respectively

SREBP1 and SREBP2 are the master transcription regulators of DNFA and DNCS pathways, respectively (49). To verify cellular dependence on SREBP1 and SREBP2 for lipid biosynthesis pathways, we transfected HT-144 cells with pooled siRNAs to deplete mRNAs encoding *SREBF1* and *SREBF2*. The transfected cells were cultured in 10% FBS, 0% FBS or 1% ITS medium conditions for two days and then assayed with RT-qPCR for DNFA (Fig. 3) and DNCS (Fig. 4) gene expression.

In the group treated with scrambled siRNA (blue bars in Figs. 3 and 4), we found that DNFA and DNCS gene expression was significantly elevated in 0% FBS

and 1% ITS medium conditions compared to 10% FBS, consistent with our observations in Fig. 2. However, the DNFA gene expression is significantly less in the *SREBF1* depleted group than in the scramble siRNA group, particularly in 0% FBS and 1% ITS medium conditions (Fig. 3A-F, statistical comparisons marked in black). The DNCS gene expression is significantly less in the *SREBF2* depleted group than in the scramble siRNA group, in 0% FBS and 1% ITS medium conditions (Fig. 4A, B, statistical comparisons marked in black). Two-way ANOVA analysis detects a significant interaction between *SREBF1/2* depletion and medium condition for DNFA and DNCS gene expression. This result provides a statistical indication that SREBP1 and SREBP2 participated in activation of lipogenic gene expression in 0% FBS and 1% ITS conditions. We further found that *SREBF1* siRNA has the greatest effect on DNFA gene expression (Fig. 3A-F), and *SREBF2* siRNA has the greatest effect on DNCS gene expression (Fig. 4A, B). LDLR appears to be regulated primarily through SREBP1 rather than SREBP2 (Fig. 4C). Our results are consistent with known roles for SREBP1 and SREBP2 in activation of DNFA and DNCS gene expression (49).

In scrambled siRNA treated groups, we observed significantly higher DNFA and DNCS gene expression in 1% ITS than in 0% FBS medium, suggesting additional influence of the ITS supplement on DNFA gene expression (Figs. 3 and 4, statistical comparisons marked in blue). In *SREBF1*-depleted group, we observed no significant increase of DNFA gene expression when comparing 1% ITS to 0% FBS condition (Fig. 3A-F, statistical comparisons marked in red). Similarly, in *SREBF2*-depleted group there is no significant elevated expression of DNCS genes, *HMGCS1* and *HMGCR*, in 1% ITS medium compared to 0 % FBS (Fig. 4A, B, statistical comparisons marked in green). Altogether, these results support the utility of the 1% ITS medium condition to study SREBP1- and SREBP2-targeted interventions to inhibit DNFA and DNCS respectively, in the absence of confounding serum lipids.

Fig 3. DNFA gene expression of HT-144 cells increases in ITS medium, and this increase is dependent upon SREBPs.

(A-F) HT-144 cells were transfected over one day with siRNAs at 50 nM concentrations for non-target control, *SREBF1* or *SREBF2*. Transfected cells were cultured in 10%, 0% FBS or 1% ITS for two more days before RT-qPCR analyses. RT-qPCR results are presented as expression of DNFA genes relative to their expression under scramble siRNA treatment (siNegative) in 10% FBS medium (set as 1 and marked with a dashed line). When a significant interaction between siRNA treatment and medium condition was detected by two-way ANOVA, individual gene expression was compared within groups by *post hoc* Tukey's multiple comparison tests. Each data point represents the mean ±SD of triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Fig 4. DNCS gene expression of HT-144 cells increases in ITS medium, and this increase is dependent upon SREBPs.

(A-E) HT-144 cells were transfected over one day with siRNAs at 50 nM concentrations for non-target control, SREBF1 or SREBF2. Transfected cells were cultured in 10%, 0% FBS or 1% ITS for two more days before RT-qPCR analyses. RT-qPCR results are presented as expression of genes relative to their expression under scramble siRNA treatment (siNegative) in 10% FBS medium (set as 1 and marked with a dashed line). When a significant interaction between siRNA treatment and medium condition was detected by two-way ANOVA, individual gene expression was compared within groups by *post hoc* Tukey's multiple comparison tests. Each data point

represents the mean ±SD of triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

ITS medium increases the level of nuclear SREBP1

To confirm that lipid-free and/or insulin-supplemented medium conditions influence SREBP1 protein production in general and its nuclear form in particular, we examined the cytoplasmic and nuclear levels of SREBP1 in HT-144 cells cultured under the three conditions (Fig. 5A). Our results revealed a dramatic increase of nuclear SREBP1 in 0% FBS and 1% ITS medium conditions. However, we did not observe a decrease of full-length SREBP1 in the cytoplasmic fraction from 0% FBS and 1% ITS medium conditions. The overall SREBP1 protein level increased in 0% FBS and 1% ITS medium conditions, which is likely due to transcriptional activation of the *SREBF1* gene in Fig. 2J.

Consistent with the increased mRNA for DNFA genes (Fig. 2D-F), there is increased production of DNFA enzymes including FASN, SCD and ACSL1 in the cytoplasmic fraction from 0% FBS and 1% ITS medium conditions. The increase of DNFA enzyme production correlates with the increase of nuclear SREBP1. We interpret this as evidence that lipid depletion combined with insulin supplement greatly enhances the abundance of the SREBP1 nuclear form and thus stimulates DNFA enzyme production.

Fig 5. Lipid depletion combined with insulin supplement yields increased expression of nuclear SREBP1.

(A) The cytoplasmic and nuclear fractions of HT-144 cells cultured in 10%, 0% FBS or 1% ITS medium were isolated for Western blot analysis. HT-144 cells in 1% ITS medium have increased levels of nuclear SREBP1 protein and lipogenic enzymes,

detected with the indicated antibodies. Histone H3 serves as the positive control for nuclear fractionation during biochemical preparation of the cell samples.

Culture in ITS medium increases SREBP1 binding at the *SCD* gene promoter

Finally, we investigated medium condition impact upon the molecular mechanism by which nuclear SREBP1 controls DNFA gene expression. We used chromatin immunoprecipitation (ChIP)-qPCR to examine the occupancy of SREBP1, transcriptional co-activator CBP (50, 51), RBP1 (the largest subunit of RNA polymerase II) and histone marker H3K27Ac (a marker for active enhancers and promoters) (52) on the promoter of DNFA gene *SCD* (Fig. 6A-D). We observed a significant increase of SREBP1 and CBP binding at the *SCD* transcription start site (TSS) in 1% ITS relative to 10% FBS (Fig. 6A, C, statistical comparisons marked in black). A corresponding slight increase of RBP1 at the *SCD* TSS site is not statistically significant, however we observe a significant RBP1 increase downstream of TSS in the gene body (Fig. 6B, statistical comparisons marked in black), consistent with active RNA polymerase II elongation (3). 1% ITS does not significantly increase the H3K27Ac signal at the *SCD* TSS nor downstream in the gene body compared to 10% FBS (Fig. 6D, statistical comparisons marked in black). However, we observed abundant H3K27Ac signals in the gene body for both 10% and 1% ITS (Fig. 6D, statistical comparisons marked in blue and red). This result suggests that the *SCD* gene body may be constitutively active, with open chromatin architecture.

Two-way ANOVA analysis detects significant interactions between gene locus and medium condition in SREBP1 and RNA polymerase II ChIP-qPCR data. This result indicates that 1% ITS significantly promotes enrichment of SREBP1 at TSS region and RNA polymerase II at gene body region. We reason that, in 1% ITS medium,

the likely transcription regulation mechanism after SREBP1 binding is promotion of the transition from RNA polymerase II pausing to active elongation (3).

Fig 6. Lipid depletion combined with insulin supplement increased SREBP1 binding at the *SCD* gene promoter.

(A-D) ChIP-qPCR analyses for enrichment of transcriptional regulation factors on the TSS and gene body region (245 bp downstream of TSS) of the *SCD* gene locus. qPCR was used to quantify chromatin immunoprecipitated with the indicated antibodies from HT-144 cells cultured in 10% FBS or 1% ITS medium. Quantification of enrichment was determined as percentage of input chromatin before immunoprecipitation. Each data point represents the mean ±SD of triplicate samples. Significant differences are indicated as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 using two-way ANOVA followed by *post hoc* Tukey's multiple comparison tests. ns, not significant.

Discussion

We report here that a serum-free and insulin-supplemented culture medium condition, 1% ITS supplemented RPMI-1640, supports DNFA and DNCS pathway activation as well as proliferation and survival of the human melanoma cell line HT-144. Under this condition, HT-144 cells proliferate while relying entirely on *de novo* lipid synthesis to meet lipid requirements. Expression of DNFA and DNCS enzymes increases significantly in cells when cultured in 1% ITS as compared with other cell culture conditions. We found that 1% ITS medium activates DNFA and DNCS gene expression through the transcription regulators SREBP1 and SREBP2, respectively. In particular, culturing cells in 1% ITS medium promoted the transcription activation of SREBP1 and accumulation of nuclear SREBP1 protein, as compared with other

427 medium conditions, and cells cultured in 1% ITS medium exhibited further increased
428 binding of SREBP1 at a DNFA gene promoter, consistent with high SREBP1-
429 dependent gene activation under this medium condition.

430 Unlike free fatty acids, free cholesterol and cholesteryl esters are only
431 minimally soluble in blood and must be transported within lipoproteins (52).
432 Lipoprotein-deficient serum (LPDS) is thus more efficient in removing cholesterol and
433 cholesteryl esters than fatty acids. LPDS has been used in lieu of full serum as an
434 activating medium for SREBPs, because it alleviates sterol inhibition (54). However,
435 LPDS contains free fatty acids that enter cells through passive membrane diffusion or
436 active transport through membrane receptors (55). LPDS has therefore also been used
437 for studies of cellular fatty acid uptake and lipid storage from extracellular fatty acids
438 (56). By contrast, ITS medium contains no external free fatty acids or lipoproteins.
439 Membrane lipids for cell proliferation in ITS medium rely entirely upon DNFA and
440 DNCS. Thus, ITS represents an attractive culture medium as compared with LPDS for
441 studying DNFA-supported proliferation and cell survival of cancer cells.

442 Our results support the idea that active DNFA is necessary for cell survival but
443 insufficient by itself for cell proliferation. Lipid depletion in 0% FBS activated DNFA
444 and DNCS gene expression, possibly due to removal of exogenous cholesterol, the
445 classic feedback inhibitor of SREBP processing and lipid synthesis (57). HT-144 cells
446 are able to persist and survive under these conditions, but become quiescent, likely due
447 to removal of growth factors in the serum necessary to support active proliferation. This
448 notion is supported by our finding that insulin present in the serum free ITS medium
449 has both metabolic and mitogenic functions in cancer cells, promoting cell survival and
450 active proliferation. It is presently unclear how DNFA is regulated to support cell
451 survival independent of insulin but may involve the AKT/GSK3 signaling pathway (58,
452 59).

Our data indicate that insulin promotes both SREBP1 processing and transcription. Nuclear SREBP1 was elevated in cells cultured in 1% ITS and 0% FBS media, compared to 10% FBS medium. However, cytoplasmic SREBP1 precursor levels remained similar in both 1% ITS and 10% FBS medium conditions. This could indicate that SREBP1 precursor levels are maintained regardless of whether a portion of SREBP1 has been processed and migrated into the nucleus. Combining these observations with the gene expression data, we believe that insulin also has a stimulative effect on *SREBF1* transcription in cancer cells, in agreement with previous findings from liver (23, 39).

DNFA is necessary for cell survival even in quiescent cancer cells that do not need membrane lipid synthesis for proliferation. In those cells, fatty acid oxidation (FAO) pathway hyperactivity has also been observed (59). DNFA and FAO are antagonistic lipid metabolism pathways that compose a futile cycle in cancer cells, but may represent a metabolic adaptation to promote cell survival under adverse (lipid-depleted) conditions (60). DNFA primarily relies on cytosolic NADPH derived from the pentose phosphate pathway (PPP) by glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME) and isocitrate dehydrogenase (IDH1), all of which are direct targets of SREBP1 (62). Cancer cells frequently promote NAPDH synthesis to support production of cellular anti-oxidants (e.g. glutathione) to counter elevated reactive oxygen species (ROS). These metabolic adaptations together may then represent a pro-survival mechanism, dependent on SREBP1.

Conclusions

In summary, we have identified and validated a serum-free and insulin supplemented (ITS) medium condition that is well suited for controlled study of

lipogenic gene activation and its mechanism of action through SREBP processing and nuclear migration, and consequent active transcription elongation in melanomas, and perhaps other cancer cell types.

Author Contributions

Conceived and designed the experiments: SW and AMN. Performed the experiments: SW. Analyzed the data: SW. Wrote the paper: SW and AMN.

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647

Figure 1.

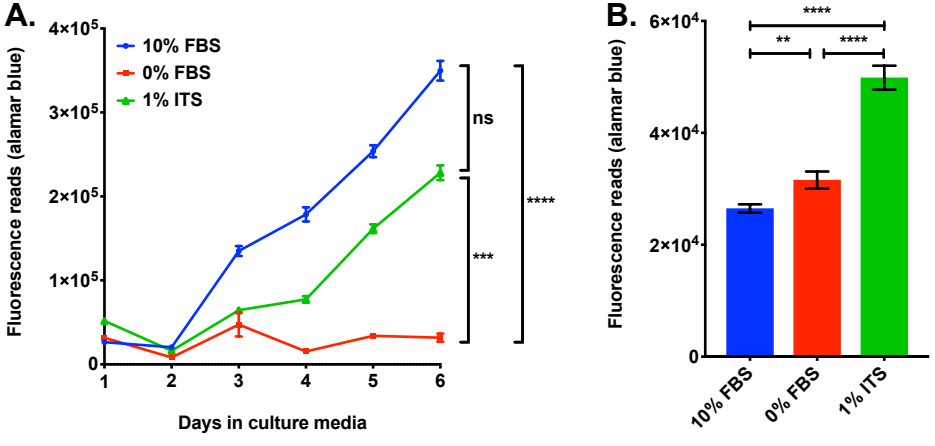

A.
4×10^5
3×10^5
2×10^5
1×10^5
0
Fluorescence reads (alamar blue)
10% FBS
0% FBS
1% ITS
ns
***
****
1
2
3
4
5
6
Days in culture media
B.
6×10^4
4×10^4
2×10^4
0
Fluorescence reads (alamar blue)
****
**
****
10% FBS
0% FBS
1% ITS

Figure 2.

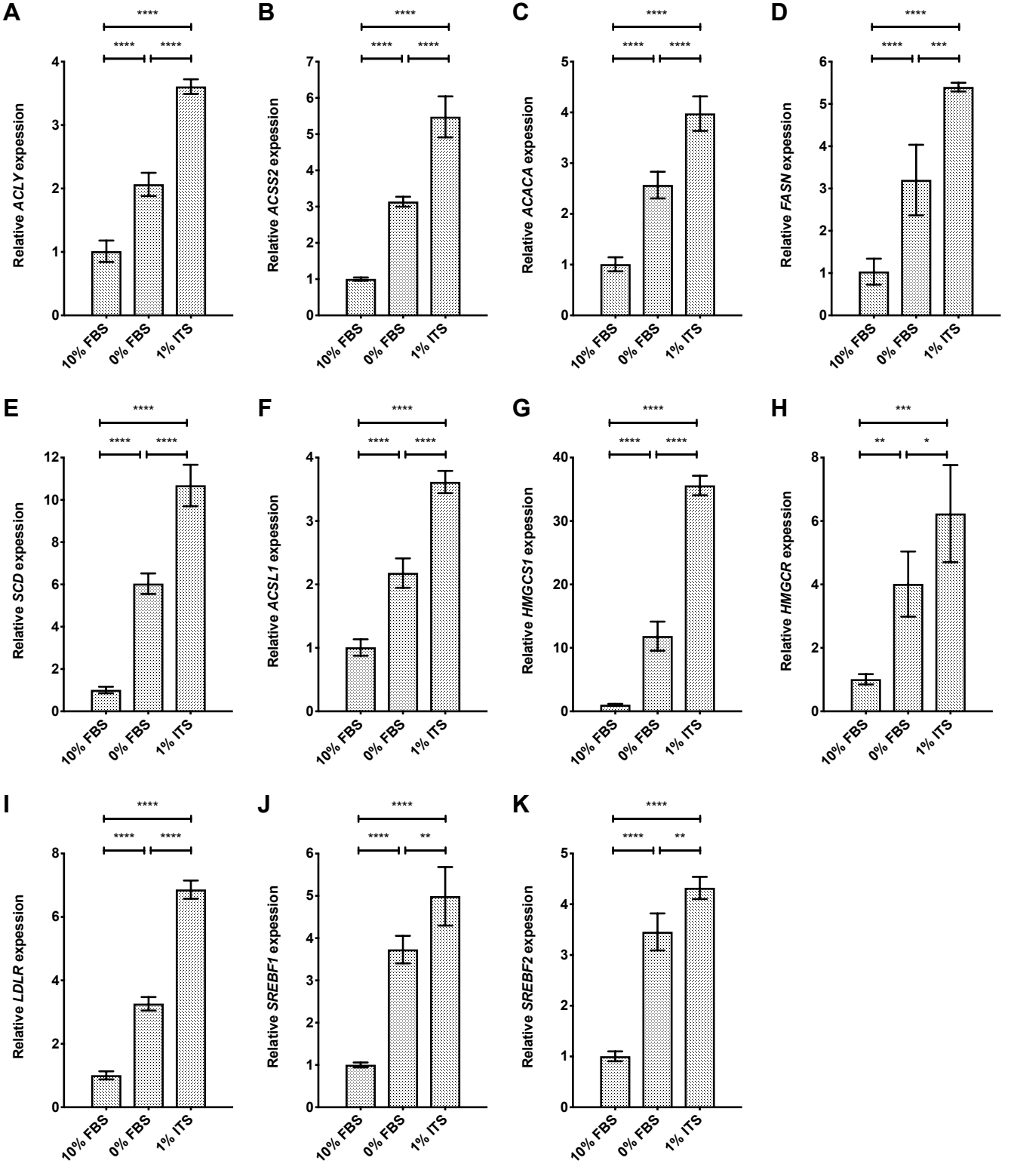

A
Relative ACLY expression
B
Relative ACSS2 expression
C
Relative ACACA expression
D
Relative FASN expression
E
Relative SCD expression
F
Relative ACSL1 expression
G
Relative HMGCS1 expression
H
Relative HMGCR expression
I
Relative LDLR expression
J
Relative SREBF1 expression
K
Relative SREBF2 expression
10% FBS
0% FBS
1% ITS

Figure 3.

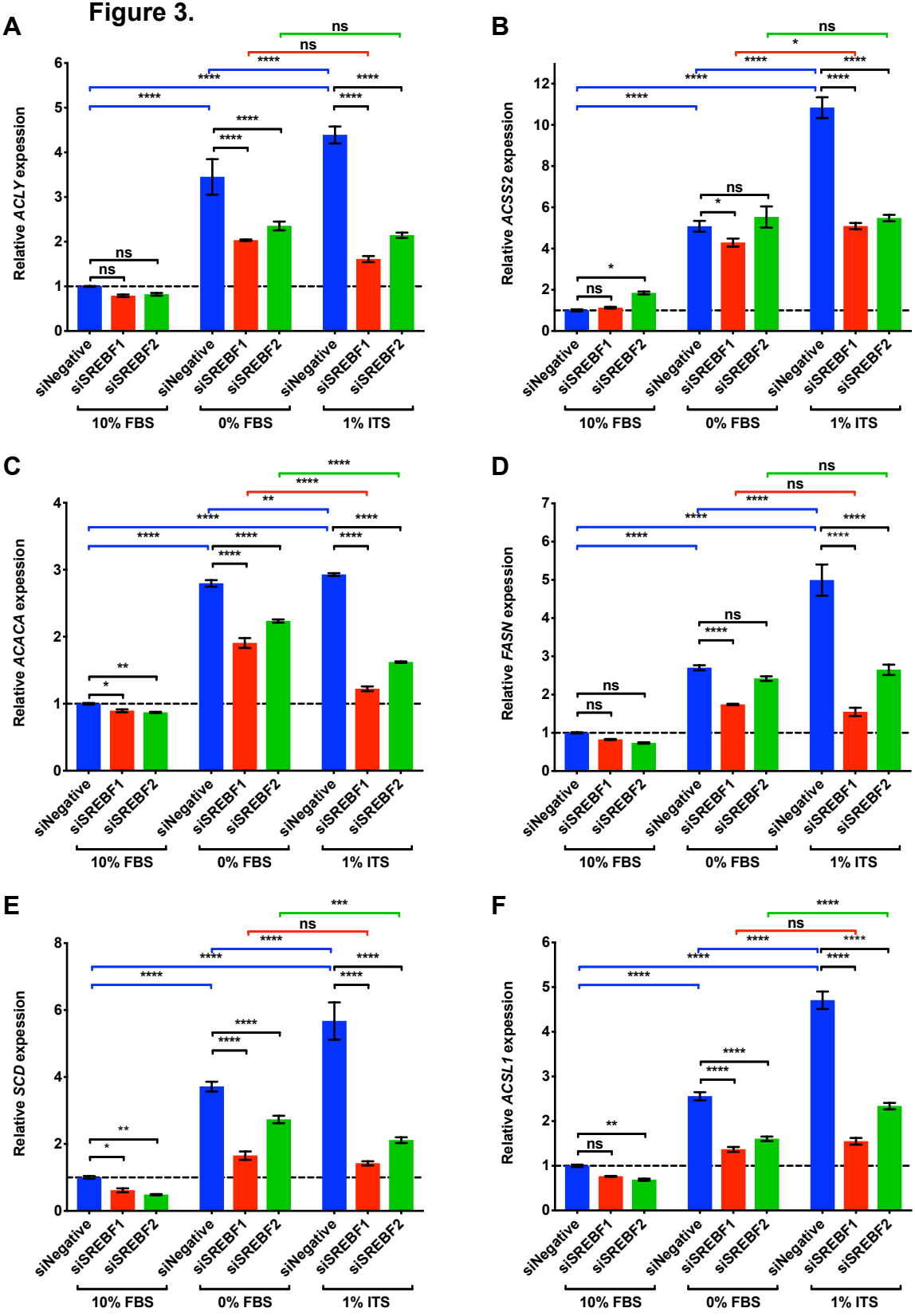

A
Relative ACLY expression
B
Relative ACSS2 expression
C
Relative ACACA expression
D
Relative FASN expression
E
Relative SCD expression
F
Relative ACSL1 expression
siNegative
siSREBF1
siSREBF2
10% FBS
0% FBS
1% ITS

Figure 4.

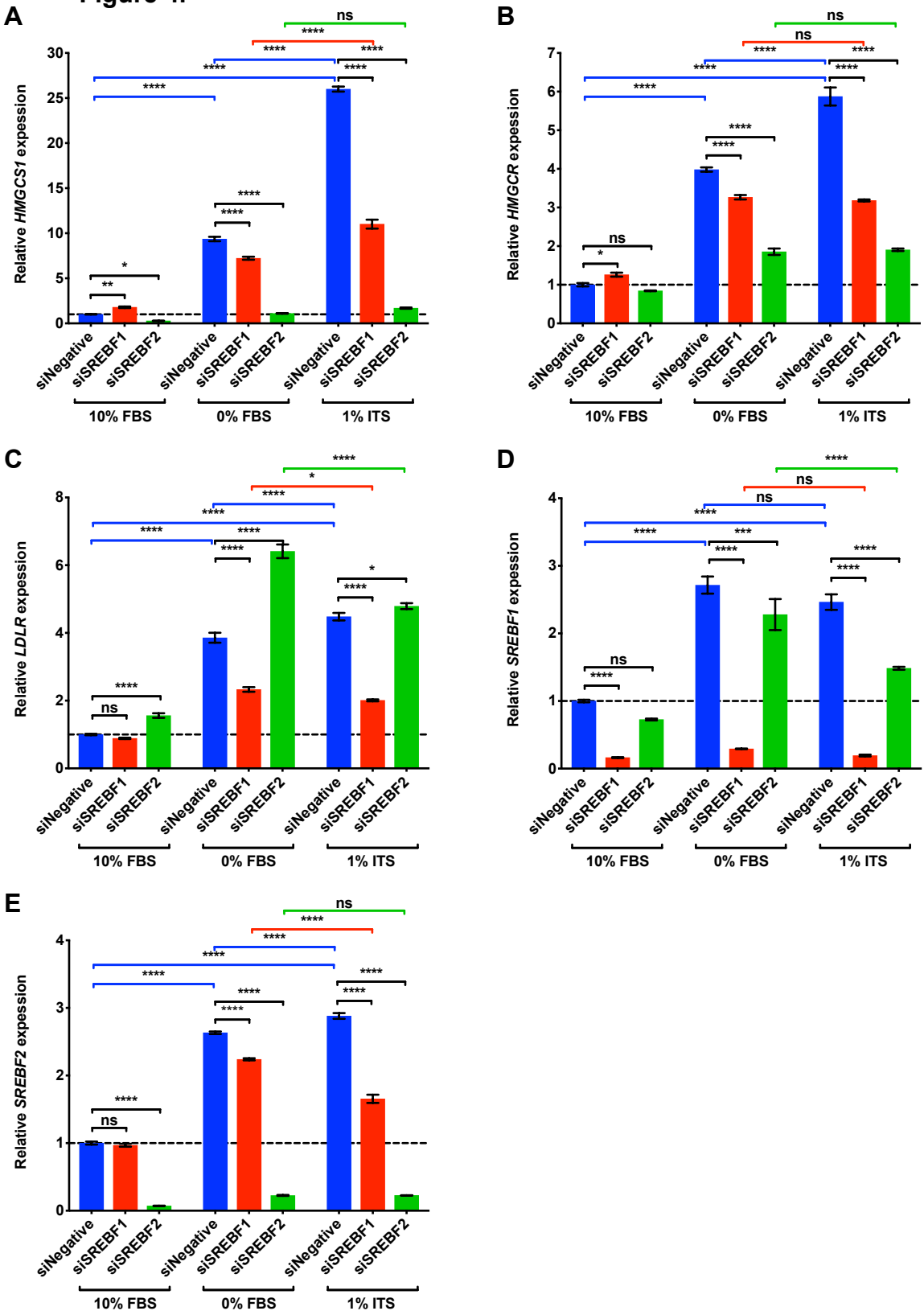

A
Relative HMGCS1 expression
B
Relative HMGCR expression
C
Relative LDLR expression
D
Relative SREBF1 expression
E
Relative SREBF2 expression
siNegative
siSREBF1
siSREBF2
10% FBS
0% FBS
1% ITS
ns
*
**
***
****

Figure 5.

A.

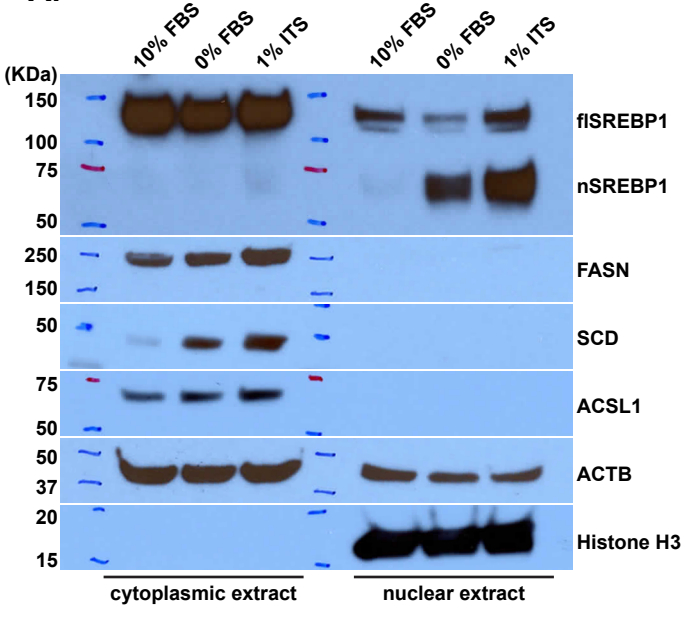
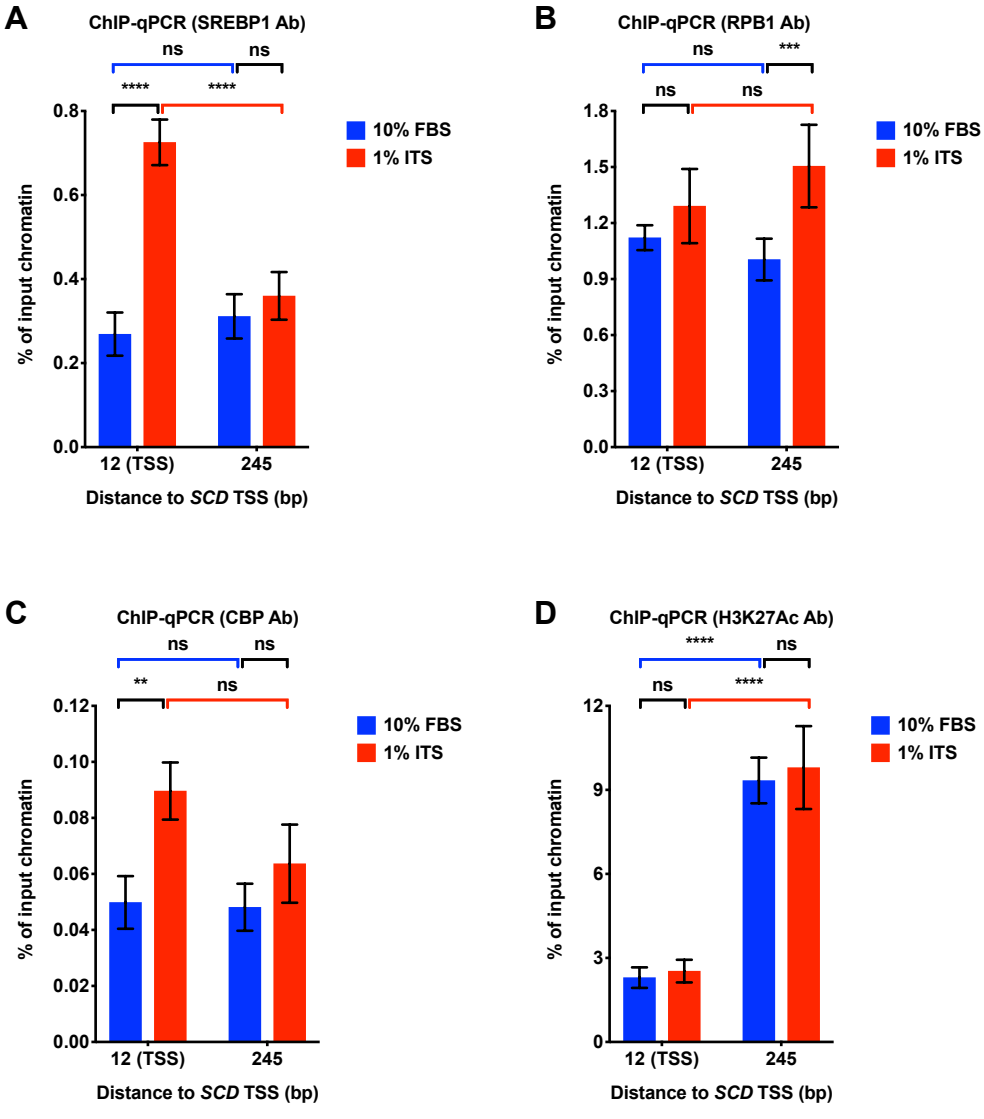

10% FBS
0% FBS
1% ITS
10% FBS
0% FBS
1% ITS
(KDa)
150
100
75
50
250
150
50
75
50
50
37
20
15
flSREBP1
nSREBP1
FASN
SCD
ACSL1
ACTB
Histone H3
cytoplasmic extract
nuclear extract

Figure 6.


A
ChIP-qPCR (SREBP1 Ab)
ns
ns
****
****
0.8
0.6
0.4
0.2
0.0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
B
ChIP-qPCR (RPB1 Ab)
ns
***
ns
ns
1.8
1.5
1.2
0.9
0.6
0.3
0.0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
C
ChIP-qPCR (CBP Ab)
ns
ns
**
ns
0.12
0.10
0.08
0.06
0.04
0.02
0.00
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
D
ChIP-qPCR (H3K27Ac Ab)
****
ns
ns
****
12
9
6
3
0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS