Y-box binding protein 1 acts as a negative regulator of stearoyl CoA desaturase 1 in clear cell renal cell carcinoma

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Received January 17, 2020; Accepted August 4, 2020

DOI: 10.3892/ol.2020.12026

Abstract. Y-box binding protein 1 (YB-1) is a regulatory protein associated with oncogenesis and poor prognosis in patients with cancer. In the cell, YB-1 functions as a DNA and RNA binding protein that promotes or suppresses expression of target genes. The cancer-promoting activity of YB-1 is mediated through its activation of oncoproteins and repression of tumor suppressor genes. Lipogenic enzyme stearoyl-CoA desaturase 1 (SCD1) drives the production of endogenous monounsaturated fatty acids (MUFAs) in cells and protects against toxic buildup of saturated fatty acids. Clear cell renal cell carcinoma (ccRCC) is often characterized by aberrantly high SCD1 expression and cytosolic accumulation of unsaturated fatty acids. In the present study, a proteomics screen of cells treated with inhibitors of SCD1 supported a potential relationship between YB-1 and SCD1. It was revealed that the presence of MUFAs led to increased protein synthesis and increased expression of high molecular weight forms of YB-1 in ccRCC cells, but not in non-tumorigenic cells. Ectopic expression of YB-1 led to decreased expression levels of SCD1 protein and mRNA in ccRCC cell lines. Conversely, targeted knockdown of YB-1 increased SCD1 mRNA abundance. Analysis of ccRCC patient data from The Cancer Proteome Atlas database showed YB-1 expression was negatively associated with survival, whereas SCD1 was associated with improved survival. These data suggested an antagonistic relationship between YB-1 and SCD1 that may influence survival of patients with ccRCC.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common form of renal malignancy (1,2). Yet, the contribution of the characteristically elevated monounsaturated fatty acids (MUFAs) to the establishment of ccRCC remains incompletely understood (3-5). It has been hypothesized that the elevated unsaturated fatty acid content of ccRCC cells is responsible for their varied response to drug therapies (6). MUFAs are produced by the enzyme stearoyl-CoA desaturase (SCD1), a protein often highly expressed in tumors (7-9). Previous studies describe unsaturated fatty acid as a stabilizer of oncoproteins that function to promote cell growth and inhibit apoptosis (10-14). This suggests that the presence of unsaturated lipids in ccRCC is necessary for growth and survival (15-21). However, there is growing evidence that demonstrates a positive correlation between MUFAs and survival in various model systems (22-25). SCD1-derived MUFAs play an important protective role in normal and transformed cells by preventing the toxic accumulation of saturated fatty acids resulting from rapid proliferation (26,27). The unsaturated lipids made by SCD1 are critical to membrane synthesis, signal transduction, and energy storage (13). Targeting SCD1 activity has been suggested as a viable strategy to combat tumor development (9,12,28). However, blocking fatty acid desaturation creates an imbalance in the ratio of saturated to unsaturated fat, leading to a cellular stress response that includes inhibition of global mRNA translation, and ultimately death in both normal and transformed cells (29,30). Therefore, despite the therapeutic potential, the relationship between SCD1 and organismal longevity, makes SCD1 a precarious drug target (22-25).

Y-box binding protein 1 (YB-1) is a phylogenetically conserved DNA and RNA binding protein that controls expression and translation of numerous genes (31). YB-1 is regulated both spatially and through posttranslational modifications. In the cytoplasm YB-1 represses the translation of mRNAs into proteins that are involved in survival and proliferation (32,33). The ability of YB-1 to repress target transcripts is regulated, in part, by tissue transglutaminase (TG2). TG2 crosslinks YB-1 into detergent-resistant oligomers, containing two or
more YB-1 monomers, in a calcium-dependent manner (34). Furthermore, transforming growth factor (TGFB-ß) also stimulates YB-1 oligomer formation, which leads to de-repression of smooth muscle actin (acta2) (35). Under certain stress conditions, YB-1 is phosphorylated and translocated to the nucleus as a transcription factor for genes that promote growth, drug resistance, and cell survival (36,37).

YB-1 is considered an oncogene because it is overexpressed in many cancers and is highly correlated with metastatic potential and patient mortality (38). While YB-1 regulates the expression of a diverse group of target genes, there is no true consensus on how YB-1 identifies its targets (39-41). We hypothesized that SCD1 is negatively regulated by YB-1 in ccRCC. The present study provides evidence of a previously unknown relationship between YB-1 and SCD1 gene expression using a combination of proteomics, bioinformatics, and molecular methods. Our data suggests a dynamic interplay between unsaturated fatty acid levels and oncogenic capacity, and highlights a relationship between protein expression and lipid levels that could have significant impact on overall survival in renal cancer patients.

Materials and methods

Materials. We obtained fatty acids (FAs) from Nu-Chek Prep, Inc.; radiolabeled S-35 methionine from Perkin-Elmer (cat. no. NEG709A500UC); SCD1 inhibitor (A939572; cat. no. 19123), Nonidet P-40 (NP-40; cat. no. 6000009) and Protein Synthesis kit (cat. no. 601100) from Cayman Chemicals; rabbit anti-YB-1 from ProteinTech; mouse anti-ß-actin from Millipore Sigma; rabbit anti-SCD1 from Cell Signal; YB-1-myc plasmid from Addgene; non-targeting control from Millipore Sigma; rabbit anti-SCD1 inhibitor (A939572; cat. no. 19123), Nonidet P-40 (NP-40). The lysate was passed through a 22 G needle 10 times while on ice followed by rotation at 4°C for 15 min. The lysate was pelleted at 21,000 x g for 10 min and the supernatant was collected. Protein concentrations were found using the BCA Gold Protein Assay kit (Thermo Fisher Scientific) and read at 490 nm. Sample lysate was mixed with loading buffer and then boiled for 5 min, and loaded onto 10% SDS-PAGE gel at 10 or 20 µg total protein per well.

Cell lines. ccRCC cells 786-O and SW156 were obtained from ATCC. Non-tumorigenic SV589 human fibroblasts were from the UT Southwestern Department of Molecular Tissue Culture Core facility. SV589 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 1.0 g/l glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin supplemented with 5% FCS (complete DMEM). SW156 and 786-O cells were grown in DMEM with 4.5 g/l glucose (DMEM-HG), 100 units/ml penicillin, and 100 µg/ml streptomycin supplemented with 5% FCS (complete DMEM-HG). Except for SV589 cells that were incubated in 8.8% CO₂, all cells were maintained at 37°C in 5% CO₂.

Lipid deprivation. Cells were plated in complete DMEM-HG at appropriate cell density for specific experimental conditions overnight. The following day, all culture media was removed and cells were rinsed one time with sterile phosphate buffered saline. Cells were replenished with delipidation media consisting of DMEM-HG supplemented with 100 units/ml penicillin, and 100 µg/ml streptomycin with 5% delipidated fetal calf serum (DFCS), and 1 µM SCD-1 inhibitor (A939572, Cayman Chemicals). Cells were incubated in delipidation media overnight and assayed for response to lipids the following day.

Cell harvest and lysis. Cells were harvested by scraping the cells and media into conical tubes and placing on ice. Following harvest, the cell suspensions were spun down at room temperature for 5 min at 800 x g. The supernatant was aspirated, and the cells were washed twice with PBS. Cells were then lysed with 100 µl of seize2 lysis buffer (25 mM Tris-HCl pH 7.2, 0.15 M NaCl, 5 µg/ml pepstatin, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µg/ml N-[N-(Acetyl-L-leucyl)-L-leucyl]-L-norleucine, 1% NP-40). The lysate was passed through a 22 G needle 10 times while on ice followed by rotation at 4°C for 15 min. The lysate was pelleted at 21,000 x g for 10 min and the supernatant was collected. Protein concentrations were found using the BCA Gold Protein Assay kit (Thermo Fisher Scientific) and read at 490 nm. Sample lysate was mixed with loading buffer and then boiled for 5 min, and loaded onto 10% SDS-PAGE gel at 10 or 20 µg total protein per well.

Stable isotope labeling of amino acids in cell culture (SILAC). Incorporation of amino acids into newly synthesized proteins in the presence of unsaturated fatty acid was measured in SW156 ccRCC cells. In brief, 150,000 cells were plated in 100 mm dishes in complete DMEM (DMEM with 4.5 g/l glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin supplemented with 5% FCS) in duplicate overnight. The following day, plates were divided equally and given either complete DMEM, 10% Delipidated FCS (DFCS) with heavy isotope of Arginine/Lysine (Heavy Medium) or complete DMEM, 10% DFCS with light isotope of Arginine/Lysine (Light Medium) for 24 h. The next day, 100 µM olate-BSA was added to cells in Heavy Medium, and 100 µM BSA alone was added to cell in Light Medium and incubated for 6 h at 37°C. Total protein was harvested using seize2 lysis buffer + protease inhibitors. For each treatment 100 µg of protein was loaded onto 16.5% Tris-Tricine BioRad precast gel (Bio-Rad, cat. no. 3450064). Gel was run only long enough to allow protein to enter resolving portion. Gel was stained with Gel-Code Blue (THERMOFISHER, cat. no. 24590), and protein band was excised with ethanol-cleaned razor and diced into 10 mm² fragments. Protein bands were sent to UT Southwestern Proteomics core facility for protein identification using a short reverse-phase Liquid Chromatography tandem mass spectrometry method.

Protein synthesis. Protein synthesis was measured by radiolabeled-methionine (S-35) incorporation into SV589 and SW156 ccRCC cells. In brief, 150,000 cells were plated in 60 mm dishes overnight. The following day cells were treated with delipidation media, DMEM +5% delipidated FCS (DFCS) + 1 µM A939572 (SCD1 inhibitor), overnight at 37°C. The following morning cells were treated with 100 µM olate-BSA or equivalent volume of 10% BSA for 4 h. Following 4-h incubation with fatty acid, cells were pulsed with 150 µCi/ml S-35 methionine media for 1 h at 37°C. After pulse, cells were washed and lysed with seize2 lysis buffer + protease inhibitors. Protein was quantitated by BCA assay and total protein was normalized across samples before performing trichloroacetic acid (TCA; cat. no. T0699, Sigma) precipitation. TCA was added to lysate at 1/4 volume of total
lysate and incubated at 4°C with agitation for 10 min. Samples were then centrifuged at 20,000 x g for 5 min. Pellets were washed twice in 200 µl of cold acetone. Pellets were dried at 95°C for 5 min and rehydrated with 200 µl scintillation fluid before transferring to labeled vials with 5 ml of scintillation fluid. Samples were read on Beckman-Coulter Scintillation counter. Protein synthesis in 786-O cells was measured by non-radioactive Protein synthesis kit from Cayman Chemical (cat. no. 601100). In brief, 500,000 cells per well were plated into 96-well black clear bottom plates and incubated at 37°C overnight. The following day, cells were kept in complete media or placed in delipidation media overnight. The following day, cells were treated with oleate, arachidonate, palmitate, or cycloheximide as negative control. Protein synthesis assay was conducted per manufacturer's instruction and read on a microplate reader at 485 nm excitation/535 nm emission.

**Proliferation assay.** 786-O ccRCC were plated into black clear bottom 96-well plates at 1,000 cells/well in triplicate in complete DMEM (100 units/ml penicillin, and 100 µg/ml streptomycin supplemented with 5% FCS). The following day cell media was exchanged for delipidation media with either 0, 10, 30, or 100 µM oleate or palmitate. Cells were placed in culture for 48 h. At the end of incubation cell growth was analyzed using Cell Titer-Glo (cat. no. G7570; Promega) according to manufacturer's instructions.

**Immunoblot analysis.** Following overnight treatment with 0, 10, 30 or 100 µM oleate or palmitate, 786-O cells were harvested and lysed as previously described. A total of 10 µg of protein was then separated by 10% SDS-PAGE. After gel electrophoresis was completed, the proteins were transferred to a 0.2-micron polyvinylidene difluoride (PVDF) membrane. All immunoblot equipment and reagents used were from Bio-Rad. The membrane was blocked with a 5% non-fat milk/PBST solution for 30 min. The membrane had anti-YB-1 antibodies (ProteinTech, 1:1,000 dilution) and proteinase K (Qiagen) atId 55°C for 2 h. DNA was eluted with 1X PBS containing a protease inhibitor cocktail. Cells were sonicated in a Diagenode Bioruptor sonicator for 30 cycles of sonication (30 sec pulses and 30 sec rest). The soluble chromatin fraction was quantitated and 100 mg of chromatin was precleared with 10 µl of Protein A Dynabeads (Invitrogen) for 2 h, then incubated overnight at 4°C with 1.5 µg of either YB-1 (9744S; Cell Signaling), or rabbit IgG (PI31887; Thermo Scientific) antibodies. The following day, 10 µl of Dynabeads were added to the chromatin-antibody mixture and incubated with rotation for 2 h at 4°C. ChIPs were washed with a low salt wash buffer (20-154; Millipore), high salt wash buffer (20-155; Millipore), and TE (20-157; Millipore). Crosslinks were reversed overnight at 65°C, followed by RNAse (Qiagen) at 37°F for 1 h, and proteinase K (Qiagen) at 55°C for 2 h. DNA was eluted using Qiaquick PCR purification kit (Qiagen) and amplified by reverse transcription-quantitative PCR (RT-qPCR). Data was analyzed using the % input method. First, input Cq was adjusted to 100% (Cq input-4.24). Results from immunoprecipitated samples were analyzed using the following calculation: 100x2^[-Adjusted input-Cq (IP)]. Fold difference was calculated against the negative control (rabbit IgG).

**RNA interference.** 150,000 cells were plated in 60 mm dishes overnight at 37°C. Following day transfection mixes were made for each experimental permutation. Functionally verified YB-1 (NM_004559) and control non-targeting siRNAs (20 µM) (Qiagen, YB-1 cat. no. SI03019191; ID: 1027415; control cat. no. 0001022076; ID: 1022076) were transfected 100 nM per dish. RNAiMax (Invitrogen) was mixed with appropriate volume of OptiMEM (Gibco) and in a separate tube the appropriate amount of each siRNA for experiment setup was added to appropriate volume of OptiMEM (Gibco), mixed and incubated at room temperature for 5 min. The two solutions were combined at equal volume, mixed and incubated at room temperature for 20 min. Plates containing cells, were washed and replenished with 1.6 ml of OptiMEM, and placed back at 37°C until transfection complexes were ready. Then 400 µl of transfection mix was added to each dish, and incubated at 37°C for 4 h, at which cells would be washed once with complete media and 4 ml of complete media would be added to cells. Cells then incubated at 37°C for 48 h.

**Chromatin immunoprecipitation assay.** 786-O cells were grown to confluence in 10 cm dishes; a final cell count of approximately 1x10⁶ cells/plate. Proteins were cross-linked to DNA using formaldehyde added directly to the culture medium at a final concentration of 1% for 10 min at room temperature. The cross-linking reaction was quenched by adding glycine to a final concentration of 0.125 M for 5 min at room temperature. The medium was then removed and cells were washed with 1X PBS containing a protease inhibitor cocktail. Then cells were scraped, pelleted and washed twice with PBS plus protease inhibitor cocktail as described above. Cells were resuspended in SDS Lysis Buffer (20-163; Millipore) plus protease inhibitor cocktail. Cells were sonicated in a Diagenode Bioruptor sonicator for 30 cycles of sonication (30 sec pulses and 30 sec rest). The soluble chromatin fraction was quantitated and 100 mg of chromatin was precleared with 10 µl of Protein A Dynabeads (Invitrogen) for 2 h, then incubated overnight at 4°C with 1.5 µg of either YB-1 (9744S; Cell Signaling), or rabbit IgG (PI31887; Thermo Scientific) antibodies. The following day, 10 µl of Dynabeads were added to the chromatin-antibody mixture and incubated with rotation for 2 h at 4°C. ChIPs were washed with a low salt wash buffer (20-154; Millipore), high salt wash buffer (20-155; Millipore), and TE (20-157; Millipore). Crosslinks were reversed overnight at 65°C, followed by RNAse (Qiagen) at 37°C for 2 h, and proteinase K (Qiagen) at 55°C for 2 h. DNA was eluted using Qiaquick PCR purification kit (Qiagen) and amplified by reverse transcription-quantitative PCR (RT-qPCR). Data was analyzed using the % input method. First, input Cq was adjusted to 100% (Cq input-4.24). Results from immunoprecipitated samples were analyzed using the following calculation: 100x2^[-Adjusted input-Cq (IP)]. Fold difference was calculated against the negative control (rabbit IgG).

**RT-qPCR and mRNA stability assay.** 24-h post transfection with siRNAs as described above, cells were re-plated into duplicate 60 mm plates for actinomycin time-course treatment. At 48 h post transfection, actinomycin treatment was initiated with 10 µg/ml of actinomycin D added to appropriate dishes. Treated samples were harvested at 0, 3, 6, and 9 h after
actinomycin treatment. Total RNA was obtained from cells by Qiazol extraction and RNeasy purification (Qiagen). YB-1 and SCD1 mRNA levels were determined using RT-qPCR using a Rotor-gene Q PCR machine. Primer sequences are presented in Table I. The data were analyzed using the $2^{-\Delta\Delta C_q}$ method and the Actin mRNA was used as an endogenous control as previously described (42). SCD1 mRNA levels were plotted against time after actinomycin D addition and fitted to a linear regression model. SCD1 mRNA half-life was calculated using the linear regression model. Data were analyzed with the unpaired Student’s t-test with Bonferroni correction and are presented as the mean ± standard deviation. Experiment was carried out in triplicate.

Statistical analysis. All experiments were performed in triplicate (n=3). An unpaired two-tailed Student’s t-test with two degrees of freedom was used to compare means of the three replicate experiments between treatments using either GraphPad Prism or MS Excel. Where appropriate, the Bonferroni correction was applied to t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Bioinformatic analysis. CcRCC Reverse Phase Protein Array (RPPA) data (n=445) was obtained from KIRC (Clear cell Renal carcinoma) dataset from The Cancer Proteome Atlas (TCPA) maintained by The University of Texas MD Anderson Cancer Center, available at http://tcpaportal.org/tcpa. The Kaplan-Meier analysis of overall survival (OS) was done using the ‘survfit’ function from Bioconductor package (https://rdrr.io/cran/survival/src/R/survfit.R) in R statistical software package (v3.4.2). Association between OS and key proteins was determined by univariable Cox proportional Hazard models. Associations between key proteins of interest were determined by multivariate pairwise analysis using Spearman ranked correlations for each pair of set of variables ($P<0.05$ implies a statistically significant marginal association at the 0.05 alpha level). Multivariate analysis was done using proportion of pairwise correlations in JMP software by SAS.

**Results**

MUFA increases protein synthesis in ccRCC. The production of MUFA is essential for basic cellular function in mammalian cells. In the production of membranes, the principal fatty acids utilized are unsaturated, oleic acids and palmitoleic acids. Previous work has demonstrated that oleic acid stabilizes the proto-oncogene β-catenin in kidney cancer cells (10). To identify other potential fatty-acid stabilized proteins, we performed proteomic analysis on cells following lipid depletion and replenishment with unsaturated fatty acids. Proteins were grouped into categories based on biological processes using PantherDB Gene Ontology Software. Several of the enrichment groupings were related to protein biosynthetic mechanisms (Fig. 1A). To confirm these data, we conducted protein synthesis analysis using either incorporation of radiolabeled-methionine or incorporation of puromycin-analogs into growing peptide chains in kidney cancer cells. In radiolabeling experiments we used ccRCC cell line, SW156, and non-tumorigenic human fibroblasts as controls. Results showed that the presence of MUFAs led to increased protein synthesis only in ccRCC cells (Fig. 1B). Next, we briefly treated an additional ccRCC cell line, 786-O, with both saturated and unsaturated fatty acids, and measured subsequent protein production. In the presence of oleic acid, protein synthesis was increased while in the presence of other fatty acids, such as saturated palmitic acid, protein production was unchanged or inhibited.

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**Table I. Primers for qPCR and ChIP.**

| Primers | Sequence (5’-3’) |
|---------|-----------------|
| **qPCR primers** | |
| β-actin forward | ATC CAC GAA ACT ACC TTC AAC TC |
| β-actin reverse | GAG GAG CAA TGA TCT GGA TCT TC |
| YB-1 forward | AAG TGA TGG AGG GTG CTG ACA ACC A |
| YB-1 reverse | GGC GTG TGC GTC GTG AAT TGA AGT T |
| SCD1 forward | GTT CCA GAG GAG GTA CTA CAA ACC TGG |
| SCD1 reverse | GTA GTT GTG GAA GCC CTC ACC CA |
| EGFR forward | GCG TTC GGC ACG GTG TAT AAG GGA CTC TGG ATC |
| EGFR reverse | GAG GCA GCC GAA GGG CAT GAG C |
| **ChIP primers** | |
| SCD1-1.5 kB Forward | AGA TCA GTA GGG TCA GAG CAT CTC AG |
| SCD1-1.5 kB Reverse | CTG CAA GCC AAT TCA CAA GAA TCG TT |
| SCD1-0.7 kB Forward | GAG GTG TCA CCA CTG TTT CTC GAG |
| SCD1-0.7 kB Reverse | TTT CTC CTT CTC AGC TCT TCT |
| SCD1-0.2 kB Forward | GGG CTG AGG AAA TAC CGG ACA C |
| SCD1-0.2 kB Reverse | CAT CTT GGC TCT CGG ATG CCG |

qPCR, quantitative polymerase chain reaction; ChIP, chromatin immunoprecipitation; YB-1, Y-box binding protein 1; SCD1, stearoyl-CoA desaturase; EGFR, epidermal growth factor receptor.
To determine other relevant biological effects of MUFA in our model of ccRCC, we performed proliferation assays on oleate-exposed cells following overnight lipid deprivation with media containing SCD1 inhibitors. The results indicate that lipid replenishment with different concentrations of oleate, led to a remarkable increase in cell growth, which is likely related to increased protein synthesis observed in previous experiments (Fig. 1D).
MUFA increases YB-1 protein expression in ccRCC. Data from the proteomics analysis indicated an enrichment of proteins involved in protein synthesis. Therefore, we searched through the proteins in our dataset to identify proteins that could potentially regulate translation and/or protein synthesis. The top protein candidate was YB-1, which could promote and suppress translation of target transcripts. To confirm that YB-1 was sensitive to the cellular levels of unsaturated fatty acid, we measured intracellular levels of YB-1 in lipid-deprived ccRCC cells following brief exposure to varying concentrations of saturated and unsaturated fatty acids. The presence of fatty acids increased the level of 50 kDa monomeric, YB-1 (p50) (Fig. 2A). However, the notable decrease of YB-1 in cells treated with 100 µM oleate was likely related to YB-1 packing into exosomes as described by others (43), while decrease of YB-1 in palmitate-treated cells was likely due to lipotoxicity (44). Interestingly, we observed a significant enrichment in high molecular weight forms of YB-1 (p100 and p150) with oleate-treated, but not palmitate-treated samples (Fig. 2A). This high molecular weight YB-1, which had been previously demonstrated to be the result of transglutaminase activity in activated fibroblasts, has decreased RNA-binding capacity compared to the YB-1 p50 monomer (34). As done in studies by Willis et al, we confirmed that endogenous transglutaminase activity in ccRCC cells was involved, to some extent, in YB-1 oligomerization by treating cultured cells with transglutaminase inhibitor, cystamine prior to immunoblot analysis (data not shown) (34). To determine whether the changes in abundance of YB-1 protein was due to increased transcription, we performed real-time quantitative PCR (qPCR) on oleate-treated ccRCC cells. We analyzed SCD1 mRNA as well, to determine if there was any negative feedback on oleic acid production during our treatment. When cells were treated with oleate-BSA, YB1 mRNA did not change, which suggested that YB1 was not a lipid-responsive gene (Fig. 2B, left). However, there was a significant decrease in SCD1 mRNA in the presence of oleate-BSA which confirmed the presence of intact negative feedback regulation in the lipogenic pathway as expected (Fig. 2B).

Overexpression of YB-1 decreases expression of SCD1 in ccRCC. While the level of MUFA appeared to affect YB-1 protein expression and oligomerization, the consequence remained unclear. We sought to determine if there was a relationship between YB-1 and SCD1. Thus, we overexpressed YB-1 in ccRCC cells using plasmid DNA, and then measured the effects on SCD1 mRNA and protein. Following transfection with YB-1, there was a decrease in SCD1 protein, as determined by immunoblot analysis (Fig. 3A). We confirmed the results of the protein analysis using qPCR, which had also shown a significant decrease in SCD1 mRNA in YB-1-transfected cells compared to controls (Fig. 3B and C). Taken together, the data illustrate that YB-1 is involved in suppressing the expression of SCD1 mRNA, however it did not indicate whether this was due to a direct or indirect effect on transcription or stability of SCD1 mRNA.

Knockdown of YB-1 increases expression of SCD1 in ccRCC. Analysis of mRNA following transfection showed that increased YB-1 expression inhibits expression of SCD1 mRNA in ccRCC. To determine whether this was dependent on inhibition of transcription or decreased mRNA stability, we first looked to decrease cellular levels of YB-1 using RNA interference. Cells were transfected with anti-sense YB1 oligos for 48 h and then total mRNA was isolated for qPCR analysis. A substantial level of knockdown was confirmed by qPCR (Fig. 4A, right panel). We also observed a corresponding increase in SCD1 mRNA, that agreed with our previous observations (Fig. 4A, left panel). To measure the effect of YB-1 on the stability of SCD1 mRNA, we looked at RNA decay rates in cells with YB1 knocked-down. First, total mRNA was assessed over a 9-h time course following treatment with the transcription inhibitor, Actinomycin D. Transcripts for YB1 remained significantly diminished compared to control at
 Levels of SCD1 mRNA increased with YB1 knockdown, while the assay control gene, epidermal growth factor receptor (EGFR) showed an increase in mRNA compared to control at each time point (Fig. 4B). To determine the effects of YB-1 on transcript stability, we analyzed the mRNA levels at each timepoint relative to t=0. Transcripts for SCD1 did not appear to change over time, while EGFR, a normally high-turnover transcript, decreased over time as expected in control transfected cells (45) (Fig. 4C). This demonstrated that SCD1 is a relatively stable transcript, even when YB-1 is decreased (Fig. 4C). This indicates that decreasing YB-1 does not affect SCD1 translation, but does lead to increased transcription of SCD1 mRNA, which can lead to the increased production of MUFA in cancer cells.

To further demonstrate that YB-1 influenced the transcription of SCD1, we decided to show the effect of MUFA on YB-1 binding to the SCD1 promoter. To identify potential binding sites and aid in the design of chromatin immunoprecipitation (ChIP) primers for the SCD1 gene promoter, we utilized the 8th release of JASPAR, the open access transcription factor binding profile database (46). We designed three sets of primers that covered roughly 2 kilobases (2 kB) of the upstream region of SCD1, with one set of primers flanking the JASPAR predicted YB-1 binding site (Fig. 5A). 786-O cells were deprived of lipids and replenished with oleate-BSA or BSA alone as previously described, before being prepared for chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) analysis as described in the methods section. Following immunoprecipitation, YB-1 was highly enriched at the predicted binding site compared to IgG control, with lower levels of signal observed along the SCD1 gene promoter. When cells were replenished with 100 µM oleate-BSA, YB-1-associated DNA is noticeably decreased (Fig. 5B). These data all together suggest that YB-1 binding to SCD1 promoter is sensitive to the level of MUFA in the cell and indicates a potential feedback mechanism between YB-1 and fatty acid levels in ccRCC.

Table II. Summary of univariable associations with overall survival in patients with ccRCC.

| Marker       | Hazard ratio | Lower CI | Upper CI | P-value |
|--------------|--------------|----------|----------|---------|
| YB-1         | 1.96         | 1.33     | 2.88     | 0.0006  |
| YB-1 pS102   | 1.77         | 1.36     | 2.3      | 0.00002 |
| SCD1         | 0.23         | 0.09     | 0.55     | 0.0009  |

ccRCC, clear cell renal cell carcinoma; YB-1, Y-box binding protein 1; SCD1, stearoyl-CoA desaturase.

YB-1 protein is negatively correlated with survival, but SCD1 improves survival. Given that YB-1 and SCD1 have documented roles in cancer, we next sought to explore the consequences of differential expression of each protein in ccRCC patients. To accomplish this, we downloaded ccRCC (KIRC) patient datasets from The Cancer Genome Atlas (47). We then sorted patients based on the availability of protein expression (reverse-phase protein arrays, RPPA) and clinical data. We determined the association between patient overall survival (OS) and each protein of interest using univariable Cox proportional Hazard models. The results of the analysis showed a strong association between each of the proteins and OS (P-value <0.05) (Table II). YB-1, and its phosphorylated form (pS102-YB-1), were both negatively associated with OS (i.e. high expression is associated with decreased survival
Figure 4. Knockdown of YB-1 increases the level of SCD1 mRNA. (A) Relative level YB-1 and SCD1 mRNA following transfection with YB-1 siRNA was measured by RT-quantitative PCR. (B) Relative abundance of YB-1, SCD1 and EGFR mRNA at each time point during actinomycin D treatment as measured by RT-qPCR. (C) Relative rate of mRNA decay was measured by comparing relative levels of SCD1 and EGFR mRNA at each time point to mRNA amount at t=0. Results are reported as mean ± standard deviation from triplicate experiments, n=3. *P<0.05 vs. Control siRNA. YB-1, Y-box binding protein 1; SCD1, stearoyl-CoA desaturase; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

Figure 5. MUFA decreases the binding of YB-1 to the SCD1 promoter. (A) The sequence of the 5'-untranslated region of SCD1 was aligned with the predicted YB-1 binding sequence obtained from JASPAR 2020 online database. (B) Binding of YB-1 to the promoter was determined by chromatin immunoprecipitation-quantitative PCR using primers to multiple regions of the SCD1 promoter (described in A). The data presented is the mean of two experiments and error bars represent the standard deviation of the sample means. YB-1, Y-box binding protein 1; SCD1, stearoyl-CoA desaturase; OA, olate.
time), the hazard ratio for YB-1 was 1.96 (95% CI: 1.33, 2.88) and the hazard ratio for pS102-YB-1 was 1.77 (95% CI: 1.36, 2.3). Conversely, high expression of SCD1 was strongly associated with improved OS, hazard ratio of 0.23 (95% CI: 0.09, 0.55). Kaplan Meir plots were generated to graphically depict these results (Fig. 6A-C). We next explored the relationship between SCD1 and YB-1, by analyzing the strength of the correlation between the expression of each protein in the...
patient data. The correlation that was observed was consistent with our molecular studies, and showed a strong negative correlation between SCD1 and YB-1 (Fig. 6D and E). Thus, the expression of YB-1 protein in patients has a negative impact on SCD1 expression which may contribute to decreased patient survival. To explain how this may work physiologically, we developed a graphical model (Fig. 7). In the model, we predict that SCD1 expression is negatively affected by YB-1. In our model, increases in YB-1 protein leads to a decrease in SCD1 mRNA synthesis and subsequently decreases cellular levels of MUFA. Conversely in our model, increased SCD1 results in increased cellular MUFA which functions to increase YB-1 oligomerization. Oligomerization of YB-1 presumably dampsens the inhibitory effect of YB-1 on SCD1, through an undefined mechanism, resulting in increased levels of SCD1 mRNA transcripts.

Discussion

In the present study, we present data indicating that YB-1 is a negative regulator of SCD1 in clear cell Renal Carcinoma (ccRCC). ccRCC is often laden with additional fat stored in the cytosol. Historically, the presence of increased fatty acids was thought to contribute to patient resistance to treatment (48). We began our work by investigating how increased fatty acids supported differential protein expression in cancer cells. We were able to demonstrate that the presence of monounsaturated fatty acid is associated with increased overall level of protein synthesis in different ccRCC cell lines compared to non-tumorigenic cells. This mechanism of increased protein expression was categorically different from that demonstrated in previous studies, where protein degradation was inhibited by the presence of MUFA (10).

Further, we showed that YB-1, an oncogene that is associated with aggressive tumor growth and poor overall survival in patients, was sensitive to the presence of monounsaturated fat. Because we saw little shift in the levels of YB1 mRNA in the presence of MUFA, we suspected that YB-1 protein synthesis was increased under lipid depletion/replenishment conditions.

Interestingly, we were able to show that YB-1 expression caused a decrease in the expression of SCD1, independent of MUFA treatment. Furthermore, we confirmed the directionality of this relationship by illustrating that YB1 knockdown resulted in increased levels of SCD1 mRNA. It was determined that SCD1 mRNA was stable even in the absence of YB-1, which ruled out alterations in mRNA stability or direct changes in translation. In absence of YB-1, EGF mRNA also showed some interesting trends in our study, however it was only included as a known control for mRNA turnover, the relationship between YB-1 and EGF will be investigated in future studies. Prompted by the effects of YB-1 knockdown on SCD1 mRNA levels, we sought to analyze interactions between YB-1 and SCD1 DNA. Chromatin immunoprecipitation data suggest that the SCD1 transcription is likely inhibited by YB-1 protein occupying a region 1.5 kb upstream of the SCD1 transcription start site, in the absence of MUFA. This indicates that YB-1 behaves as a negative regulator of SCD1 transcription when fatty acids levels are below a certain threshold.

Finally, as YB-1 has been demonstrated to drive cancer growth in patients, we became interested in identifying correlations in expression between YB-1 and SCD1 protein in renal cancer patients. Our bioinformatic analysis of several hundred patients' records, confirmed our in vitro findings to show that YB-1 is negatively associated with SCD1 at the protein level. However, the most interesting finding was that SCD1 had a largely positive association with patient survival. Meaning that in our patient population, individuals whose tumors had increased levels of SCD1 protein had a statistically significant increase in 5-year survival compared to patients with less tumor associated SCD1 protein. While this may conflict with previous cancer studies looking at SCD1 mRNA levels and limited immunohistochemical assessments, this agrees with previous studies in yeast, nematodes, and human cells that demonstrated a general benefit to cellular health and longevity in the presence of MUFA, such as oleate (8,23,25,49,50). To the best of our knowledge, this is the first report that implicates YB-1 as a regulator of lipogenic genes. Furthermore, this is one of the first reports to suggest that SCD1 expression may antagonize the development of more aggressive cancer phenotypes in patients. A similar study of SCD1 in CML also questions the only role of SCD1 as an oncogene, and demonstrates its potential as a tumor suppressor (51). Based on our findings, it appears that investigating the potential benefit of measuring YB-1 and SCD1 gene expression in patients should be considered, while implementation of any SCD1-targeted therapies should be done with considerable precaution.

Acknowledgements

The authors would like to acknowledge the support of Ms. Jara McLaren (Simmons Laboratory, UMN Medical School) for providing an early review of the literature regarding function and localization of YB-1 in the context of cancer; Mrs. Barbara Perslin (Department of Biomedical Sciences, UMN Medical School) for administrative support in the ordering of supplies and reagents; Dr Benjamin Clarke (UMN Medical School) for providing the tissue culture facilities for the work done for this manuscript; Mrs. Shannon RedBrook (Clarke Lab) for technical support regarding use of the Clarke laboratory tissue culture facility; and Dr Jean Regal (Department of Biomedical Sciences, UMN Medical School) for orchestrating critical staff support to the project.

Funding

This work was supported by the Whiteside Institute for Clinical Research and by the National Institutes of Health's National Center for Advancing Translational Sciences (grant no. UL1TR002494). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health's National Center for Advancing Translational Sciences.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.
Authors' contributions

EI, SF, BC, KR, TC, RM, LB and GES designed studies and analyzed data. EI and GES wrote the manuscript. EI, SF, BC, KR, TC, RGM, LB and GES performed experiments. GES supervised the entire study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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