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Article

Under Normoxia Simvastatin Shifts Lipid Metabolism of B16.F10 Melanoma Cells to Support Their Aggressiveness

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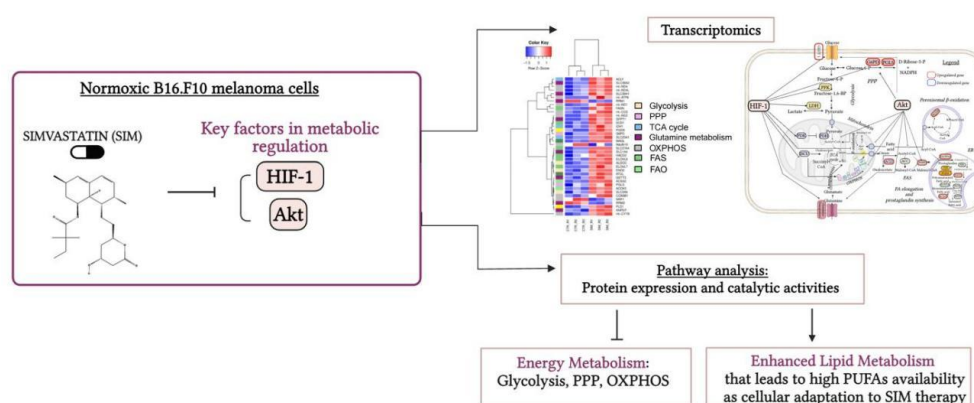
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Abstract: Our previous studies demonstrated a tight connection between simvastatin (SIM) inhibition of B16.F10 murine melanoma cell proliferation *in vivo* and *in vitro* and strong suppression of the subunit α of hypoxia inducible factor 1 (HIF-1) (HIF-1 α) production in these cancer cells. Since the translation of HIF-1 α is modulated by signaling pathways coordinated by Akt, SIM effects on the link between these two regulatory factors and cancer cell metabolism under normoxia was investigated, which to our knowledge have never been described before. To achieve the proposed aim, SIM effects on cell metabolism were analysed at mRNA (via RNA-seq and RT-qPCR) as well as protein level (via western blot and catalytic activity of the key enzymes). Our data suggested that SIM might orientate glucose transport and metabolism to ensure replenishment of tricarboxylic acid (TCA) cycle activity. Moreover, it seems that SIM enhanced biosynthetic role of TCA over its energy role to further support synthesis of lipid-derived signaling molecules. Thus, under normoxia SIM treatment orientated lipid metabolism to stimulate isoprenoid and prostaglandin synthesis which might favor melanoma cell survival and aggressiveness.

Abbreviations: 15-prostaglandin dehydrogenase (1.15-PGDH), acetyl-Coenzyme A acetyl-transferase 2 (ACAT2), acyl-Coenzyme A oxidase 3 (ACOX3), protein kinase B (also known as serine/threonine-specific protein kinases) (Akt), aldolase C (ALDOC), adenosine monophosphate (AMP), alanyl aminopeptidase (ANPEP), adenosine diphosphate (ADP), adipose triglyceride lipase (ATGL), ATP citrate lyase (ACLY), citrate synthase (CS), differentially expressed genes (DEGs), 5,5'-Dithiobis-2-Nitrobenzoic Acid (DTNB), eukaryotic translation initiation factor 4A1 (eIF4A1), enolase 2 (Eno2), endoplasmic reticulum (ER), electron transport chain (ETC), elongation of long chain fatty acids family (ELOVL), fatty acids elongation (FAE), β -oxidation (FAO), fatty acids synthesis (FAS), fatty acid synthase (FASN), fatty acids (FAs), farnesyl diphosphate synthase (FDPS), forkhead box M1 (FOXO1), glucose 6-Phosphate Dehydrogenase (G6PD), glutamine fructose-6-phosphate transaminase 1 (GFPT1), glucose transporter 1 (GLUT1), L- γ -glutamyl-p-nitroanilide (GPNA), glutathione S-transferase theta 3 (GST theta 3), 3-hydroxy-acyl-CoA dehydrogenase (HACD2), hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CR), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMG-CS1), heat shock protein 90 (HSP90), isopentenyl-diphosphate isomerase (IDI1), isocitrate dehydrogenase 1 (IDH1), insulin-induced gene protein 1 (INSIG-1), insulin-induced gene protein 2 (INSIG-2), Lactate Dehydrogenase (LDH), monoacylglycerol lipase (MAGL), monounsaturated fatty acids (MUFAs), mitochondrially encoded NADH dehydrogenase 1 (mt-ND1), mevalonate (diphospho) decarboxylase (MVD), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), NADH Oxidoreductase Subunit B10 (Ndufb10), nuclear respiratory factor 1 (NRF1), oxidative phosphorylation (OXPHOS), pyruvate dehydrogenase (PDH), PDH kinase-1 (PDK-1), phosphofructokinase 1 (PFK1), 6-phosphogluconolactonase (PGLS), PTEN induced putative kinase 1 (PINK1), prostaglandin D2 synthase (PGDS), protein kinase B (PKB), phospholipase D1 (PLD1), pentose phosphate pathway (PPP), polyunsaturated fatty acids (PUFAs), ribosomal protein S6 (RPS6), ribosomal protein S6 kinase polypeptide 2 (Rps6kb2), ribonucleotide reductase 1 (RRM1), ribonucleotide reductase 2 (RRM2), simvastatin (SIM), stearyl

Co-A desaturase 1 (SCD1), saturated fatty acids (SFA), solute carrier family 1 glutamate transporter, member 4 (SLC1A4), solute carrier family 2, member 1 (GLUT1) (SLC2A1), facilitated glucose transporter solute carrier family 2, member 6 (GLUT6) (SLC2A6), solute carrier family 25 member 1 (SLC25A1), solute carrier transporter member 4 (SLC27A4), solute carrier family 38, member 1 (SLC38A1), squalene epoxidase (SQLE), sterol regulatory element binding proteins (SREBP1), tricarboxylic acid cycle (TCA), unsaturated fatty acids (UFAs).

Keywords: simvastatin; normoxia; hypoxia inducible factor 1; transcriptomics; unsaturated fatty acids; prostaglandins



Graphical abstract. Simvastatin favored Lipid Metabolism in B16.F10 Melanoma Cells via modulation of the constitutive level of HIF-1. Abbreviations: Akt: Protein kinase B, FAO: Fatty Acid Oxidation, FAS: Fatty Acid Synthesis, HIF-1: Hypoxia-Inducible Factor 1, HMG-CoA: 3-Hydroxy-3-Methylglutaryl-Coenzyme A, HMC-CR: HMG-CoA Reductase, OXPHOS: Oxidative Phosphorylation, PPP: Pentose Phosphate Pathway, PUFAs: Polyunsaturated Fatty Acids, SIM: Simvastatin, TCA Cycle: Tricarboxylic Acid Cycle. Created in BioRender. Gabriela, G. (2024) BioRender.com/j03y911

1. Introduction

Statins have antitumor effects when administrated in doses 100-500 times higher than those needed to lower cholesterol biosynthesis (Coimbra et al, 2010; Alupei et al, 2014; Licarete et al, 2017; Porfire et al, 2014; Alupei et al, 2015). Thus, our data proved that simvastatin (SIM), a lipophilic statin strongly inhibited B16.F10 murine melanoma cell proliferation *in vivo* and *in vitro* via strong suppression of the expression of subunit α of hypoxia inducible factor 1 (HIF-1 α) (Alupei et al, 2014; Licarete et al, 2017; Alupei et al, 2015) under hypoxic (Licarete et al, 2017) as well as normoxic conditions (Alupei et al, 2014). Notably, hypoxia-stabilized expression of HIF-1 α is essential for regulation of multiple cancer-related metabolic pathways such as activation of metabolic genes involved in increasing glycolysis, fatty acid synthesis (FAS), pentose phosphate pathway (PPP), and nucleotide biogenesis (Kumar et al, 2013; DeBerardinis and Chandel, 2016; Munir et al, 2019; Shiratori et al, 2019). It is known that one of the hallmarks of cancer cells remains the HIF-1-regulated metabolism reprogramming which supports cell growth, survival, metastasis, and also resistance to therapies (Warburg, 1956; Warburg 1925; Phan et al, 2014; Cazzaniga et al, 2015; Pavlova and Thompson, 2016). Thus, HIF-1 suppresses pyruvate dehydrogenase (PDH), *via* activation of PDH kinase-1 (PDK-1) expression, resulting in inhibition of the mitochondrial metabolism (tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS)), which ensures cancer cell survival under hypoxia (Kumar et al, 2013; DeBerardinis and Chandel, 2016; Munir et al, 2019; Shiratori et al, 2019). Besides the role of HIF-1 to ensure tumor survival and progression under hypoxic stress conditions, normoxic expression of HIF-1 α in melanoma tumors is associated with increased aggressiveness of these cancer cells (Mills

et al, 2009; Kuphal et al, 2010; Alupei et al, 2014). In tight connection with our earlier observation about the suppressive effect of SIM on normoxic HIF-1 α expression (Alupei et al, 2014), we aimed to explore if by interfering with HIF-1 α key metabolic regulator in normoxic conditions, the cell's metabolism was influenced. RNA-seq revealed expression changes induced by SIM in genes involved in energy and biosynthesis metabolic pathways. To validate these data, we evaluated the expression and catalytic activities of several proteins such as fatty acid synthase (FASN), stearylCoA desaturase 1 (SCD1) and phosphofructokinase 1 (PFK1). Our results indicated that low-dose SIM treatment (at IC50 value) influenced B16.F10 melanoma cells metabolism to adapt and become resistant to the treatment. The metabolism was redirected towards the synthesis of fatty acids (FAs), mainly favoring unsaturated fatty acids (UFAs) abundance. This metabolic shift could potentially result in elevated prostaglandin synthesis, bioactive lipids with various signaling roles in the progression of cancer (Wang and Dubois, 2010; Wang et al, 2015; Wang et al, 2022; Ferreira et al, 2018; Yoshida et al, 1998; Gomes et al, 2018).

2. Materials and Methods

Materials and Methods should be described with sufficient details to allow others to replicate and build on published results. Please note that publication of your manuscript implicates that you must make all materials, data, computer code, and protocols associated with the publication available to readers. Please disclose at the submission stage any restrictions on the availability of materials or information. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited.

Research manuscripts reporting large datasets that are deposited in a publicly available database should specify where the data have been deposited and provide the relevant accession numbers. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.

Interventionary studies involving animals or humans, and other studies require ethical approval must list the authority that provided approval and the corresponding ethical approval code.

2.1. Cell line and Culture Conditions

B16.F10 murine melanoma cells (ATCC; CRL-6457) were cultured in DMEM (Lonza Group, Basel, Switzerland CH) as a monolayer at 37°C in 5% CO₂ and 20% O₂ atmosphere, as previously described (Alupei et al, 2014; Patras et al, 2021).

2.2. SIM Solutions

SIM was purchased from Sigma-Aldrich Chemie GmbH, Germany. SIM IC50 (3.5 μ M), as determined in our previous study (Alupei et al, 2014) was prepared from the stock solution of 24 mM SIM into the culture medium shortly before use. Cells were incubated with 3.5 μ M SIM for 24h.

2.3. RNA-Seq

B16.F10 cells were seeded as 1x10⁶ cells/flask and treated with 3.5 μ M SIM for 24h at 37°C. Then, the RNA isolation was performed using peqGOLD Total RNA isolation kit (PeqLab, Erlangen, DE), including the DNase digestion treatment of the lysates (peqGOLD DNase I Digest Kit, PeqLab, Erlangen, DE), to avoid DNA contamination, according to the manufacturer's instructions (Hemmrich et al, 2010). The RNA was eluted with 50 μ l of RNase-free water. The quantification of total RNA was performed by Invitrogen Qubit 4 Fluorometer (Thermo Scientific, MA, USA) with Broad Range (BR) Assay kit (Thermo Scientific, MA, USA), while RNA quality was determined using NanoDrop 1000 (Thermo Scientific, Ma, USA) according to the manufacturer's protocol (Zhang et al, 2020). To remove rRNA from the total RNA Ribo-Zero rRNA removal beads (Illumina, Inc.) were used. Further, to generate strand-specific cDNA libraries, TruSeq Stranded Total RNA (Illumina, Inc.) was used followed by quality control procedure (Qubit 2.0 Fluorometer, Thermo Scientific, USA; Agilent 2100 bioanalyzer, Argilent Technologies, USA). The qualified libraries were then subjected

to sequencing by high-throughput Illumina NovaSeq 6000 sequencing platform, PE 150 (Illumina, Inc.). Three biological replicates were used for each experimental group. Library construction and sequencing were performed by Novogene Corporation (UK).

2.4. RNA-seq Data Analysis

The clean obtained sequencing data were mapped to the mouse reference transcriptome by HISAT2 software (v2.0.5) (Kim et al, 2015). For the estimation of transcript abundance of coding genes as Fragments Per Kilobase of transcript per Million mapped reads (FPKM), StringTie-eB software (v1.3.1) (Pertea et al, 2015) was used, then the differentially expressed transcripts were identified by Ballgown package 2.22.0 (Robinson et al, 2010; Pertea et al, 2016). To measure the level of expression of each gene in SIM-treated group in comparison with its expression in control group, fold regulation (FR) values were calculated. Benjamini-Hochberg method was applied to adjust p-values for multiple testing (Benjamini and Yekutieli, 1995; Benjamini and Yekutieli, 2001). Genes with a FR cutoff of ± 1.5 , a *p-value* less than 0.05 and a false discovery rate (FDR) of 20% were considered differentially expressed (Barbariga et al, 2019; Floares et al, 2011). Furthermore, pathway annotation of differential genes was performed in KEGG database (<https://www.genome.jp/kegg/pathway.html>), testing the statistical enrichment of mRNAs with KOBAS software (v.3.0). Hierarchical clustering of expression Z-scores of differentially expressed genes (DEG) considered of interest was done in R, using heatmap.2 function. Z-scores were calculated for each row (each DEG) as follows, $z=(x-\mu)/\sigma$, where *x* is the count value of the gene in a replicate, μ the mean of all count values of this gene in all replicates, and σ the corresponding standard deviation (SD) (Weinguny et al, 2020).

2.5. Quantitative Real-Time PCR (RT-qPCR)

RT-qPCR analysis was used to validate RNA-seq data. Total RNA (1 μ g) for each group was reverse-transcribed into cDNA, using iScriptTM Reverse transcription Supermix (#1708841, Bio-Rad Laboratories (Pacific) Pty Ltd, Gladesville, Australia) in a total volume of 20 μ l. The complete reaction mix was incubated at 25°C for 5 min for priming, followed by reverse transcription at 46°C for 20 min and finally at 95°C for 1 min for reverse transcriptase inactivation. Real-time quantitative PCR was performed using SsoFastTM EvaGreenR Supermix (#172-5200, Bio-Rad Laboratories (Pacific) Pty Ltd, Gladesville, Australia). cDNA (1 μ l) was amplified with 500 mM specific primers. The primers sequences are mentioned in **Table 1** (purchased from Eurogentec, Seraing, Belgium). The following setup was used for the amplification: enzyme activation at 95°C for 30 sec, 40 cycles of denaturation at 95°C for 5 min, and annealing at 55°C for 5 sec, followed by 65°C-95°C for 5 sec (in 0.5°C inc). PCR and subsequent 1% agarose gel electrophoresis were used for validation of primer specificity (**Figure 1**). All reactions were performed in CFX96TM Real-Time System (C1000 Touch Thermo Cycler, Bio-Rad Laboratories (Pacific) Pty Ltd, Gladesville, Australia) according to manufacturer's instructions. Relative mRNA expression was quantified using the $\Delta\Delta$ Ct method (Ct is threshold cycle) and normalized to β -actin mRNA as an internal control (Livak and Schmittgen, 2001).

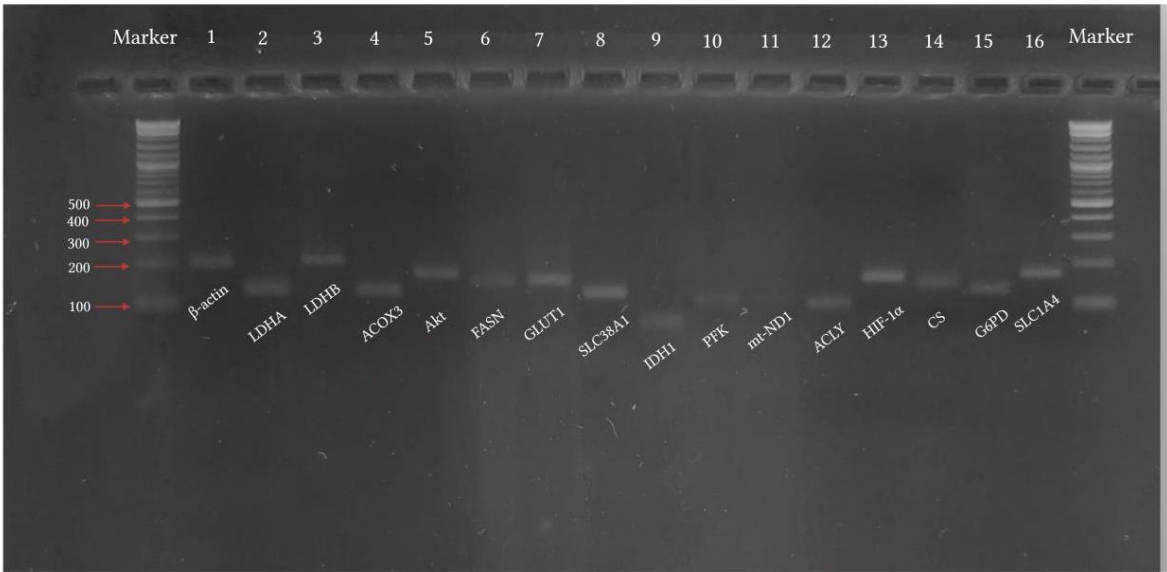


Figure 1. Validation of primer specificity by agarose electrophoresis of PCR products. **Abbreviations:** ACLY: ATP citrate lyase; ACOX3: acyl-CoA oxidase 3; b-actin: beta-actin; CS: citrate synthase; FASN: fatty acid synthase; GLUT1: glucose transporter type 1; G6PD: glucose-6-phosphate dehydrogenase; HIF-1α: hypoxia-inducible factor 1 alpha; IDH1: isocitrate dehydrogenase 1; LDHA: lactate dehydrogenase A; LDHB: lactate dehydrogenase B; Akt: protein kinase B; mt-ND1: mitochondrial NADH dehydrogenase subunit 1; PFK: phosphofructokinase; SCD1: stearoyl-CoA desaturase 1; SLC1A4: solute carrier family 1 member 4; SLC38A1: solute carrier family 38 member 1.

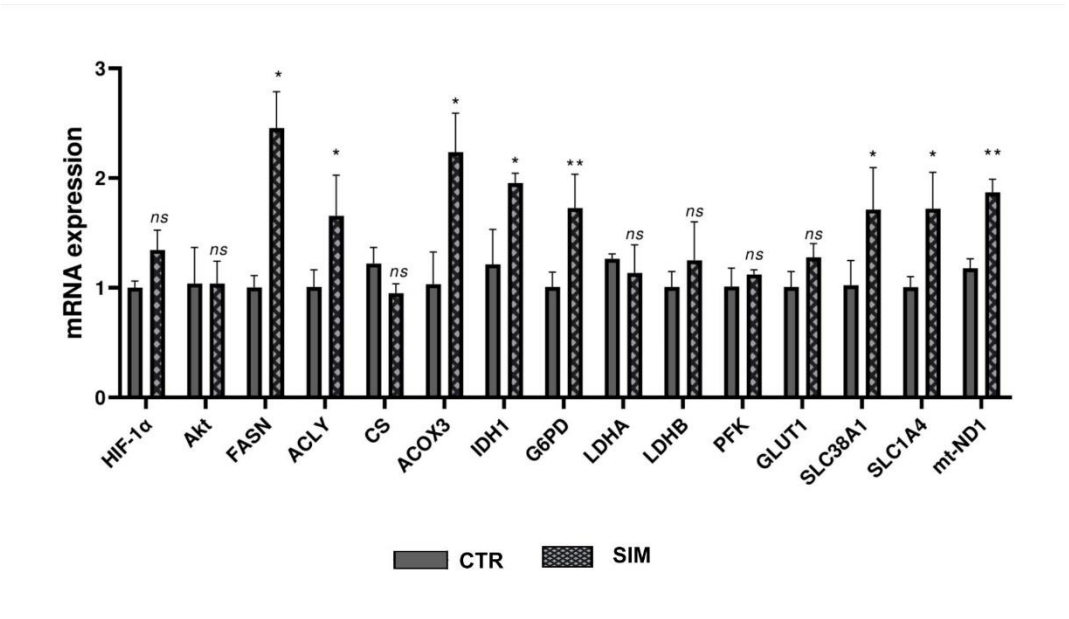


Figure 2. Relative expression of genes involved in metabolic pathways assessed by RT-qPCR. The figure presents the relative expression of 15 reference genes involved in metabolic pathways in B16.F10 melanoma cells treated with SIM, compared to the gene expression in the untreated group. β-actin served as a housekeeping gene in the qPCR analysis. The data are expressed as mean ± SD. At least three replicates were used for the quantification of each gene. Statistical significance was assessed using Student's T-test. ns: not significant; * $p < 0.05$; ** $p < 0.01$. **Abbreviations:** ACLY: ATP citrate lyase; ACOX3: acyl-CoA oxidase 3; b-actin: beta-actin; CS: citrate synthase; FASN: fatty acid synthase; GLUT1: glucose transporter type 1; G6PD: glucose-6-phosphate dehydrogenase; HIF-1α: hypoxia-inducible factor 1 alpha; IDH1: isocitrate dehydrogenase 1; LDHA: lactate dehydrogenase A; LDHB: lactate dehydrogenase B; Akt: protein kinase B; mt-ND1: mitochondrial NADH

dehydrogenase subunit 1; PFK: phosphofructokinase; SCD1: stearoyl-CoA desaturase 1; SLC1A4: solute carrier family 1 member 4; SLC38A1: solute carrier family 38 member 1.

Table 1. Primers sequences for targeted mouse genes used for RT-qPCR.

	Gene	Description	Primers sequence (5' to 3')	Accession number
1	HIF-1α	Hypoxia inducible factor 1α	F CATAAAGTCTGCAACATGGAAGGT R ATTTGATGGGTGAGGAATGGGTT	NM_001313919.2
2	Akt	Protein kinase B	F ATCCCCTCAACAACCTTCTCAGT R CTTCGCTCCACTCTTCTCTTTC	NM_011785.4
3	FASN	Fatty acid synthase	F CCCTTGATGAAGAGGGATCA R ACTCCACAGGTGGGAACAAG	NM_007988.3
4	ACLY	ATP citrate lyase	F TTCGTCAAACAGCACTTCC R ATTTGGCTTCTTGAGGGTG	NM_001199296.1
5	CS	Citrate synthase	F TTGGGAGCCAAGAACTCATC R TCTGGCTGCTCCTTAGGTA	NM_026444.4
6	ACOX3	Acyl-Coenzyme A oxidase 3	F ACCCACGGATAAGGAAGAGA R GAGAAGTGGTCCAAGGCATAG	NM_030721.4
7	IDH1	Isocitrate dehydrogenase 1	F TTCCAGGAGATCTATGACAAGAAA R TGAGCCTGTGTTCATAGCAGA	NM_001111320.1
8	G6PD	Glucose-6-phosphate dehydrogenase	F CACAGTGGACGACATCCGAAA R AGCTACATAGGAATTACGGGCAA	NM_008062.3
9	LDHA	Lactate dehydrogenase A	F AACATCTCACTCCCACAGC R CTCACAGGGGTAATCGAAGC	NM_010699.2
10	LDHB	Lactate dehydrogenase B	F TGCCTCCGTTGCAGATGAT R TTTCCGAGTCTGGAGGAACAA	NM_008492.3
11	PFK	Phosphofructokinase	F CAGATCAGTGCCAACATAACCAA R CGGGATGCAGAGCTCATCA	NM_001163487.1
12	GLUT1	Glucose transporter 1	F CTGTCATCAACCGCAAC R CTTCTTCTCCGCATCATCT	NM_011400.4
13	SLC38A1	Solute carrier family 38, member 1	F CCTTCACAAGTACCAGAGCAC R GGCCAGCTCAAATAACGATGAT	NM_134086.4
14	SLC1A4	Solute carrier family 1, member 4	F GGCATCGCTGTTGCTTACTTC R CGAGGAAAGAGTCCACTGTCT	NM_018861.3
15	mt-ND1	Mitochondrially encoded NADH dehydrogenase 1	F CTAATCGCCATAGCCTTCCTAA R GTTGTTAAAGGGCGTATTGGTT	NM_001001130.3
16	ACTB	Beta actin	F CAGGTCACTCAATTGGCAA R AGGTCTTTACGGATGTCAAC	NM_007393.5

2.6. Cell Lysates Preparation

Prior to inducing cell lysis, the B16.F10 cell monolayers were washed with PBS and the viable adherent cells were detached with Trypsin 1x (5 min incubation), then centrifuged at 260 x g for 5 minutes. Thereafter, the obtained pellets were resuspended in 250 µl lysis buffer as described previously (Rauca et al, 2018). The homogenates were kept on ice for 30 minutes, followed by centrifugation at 4°C at 12 000 x g for 10 minutes. The supernatants were collected and stored at -80°C before further molecular measurements (Luput et al, 2018). Bradford assay was performed to determine the total protein concentration in the cell lysates (Sigma-Aldrich; Merck KGaA) (Bradford, 1976; Alupei et al, 2014; Rauca et al, 2018).

2.7. Assessment of Lactate Dehydrogenase (LDH) Activity

To assess the LDH activity, the decrease of nicotinamide adenine dinucleotide (NADH) absorbance at 340 nm was monitored in 10 µl of cell lysates. The variation of absorbance is directly proportional to the LDH activity, caused by conversion of NADH to NAD⁺, while lactate is transformed into pyruvic acid. The determination was performed using Jasco V-530 UV-VIS spectrophotometer, following the protocol described by Huijgen et al, 1997. The values were represented as catalytic activity mU/mg protein. Three independent experiments were performed.

2.8. Determination of PFK1 Activity

To test the activity of the glycolysis rate-limiting enzyme PFK1, Phosphofructokinase Assay Kit (MAK093, Sigma-Aldrich Chemie GmbH, Germany) was used. The principle of the assay is based on the coupled conversion of fructose-6-phosphate to fructose-1,6-diphosphate by the enzyme mix, resulting adenosine diphosphate (ADP) which is further converted to adenosine monophosphate (AMP) and NADH (Alice et al, 2002). Then, NADH reduces a colorless probe, in a way dependent of the PFK activity. The obtained values were represented as miliunits (mU) of PFK1 activity/ml after measuring absorbance at 450 nm. Three independent experiments were performed.

2.9. Glucose 6-Phosphate Dehydrogenase (G6PD) Activity Assay

To evaluate G6PD activity, the first and rate-limiting enzyme of the PPP, 1×10^6 cells were homogenized in 100 μ l ice-cold PBS, for each experimental group. The measurements were performed using G6PD activity assay kit (MAK015, Sigma-Aldrich Chemie GmbH, Germany) according to the supplier's instructions. Thus, 20 μ l of homogenate/sample was used. After the oxidation of glucose-6-phosphate by the enzyme mix, a colorimetric product was generated, and the intensity was monitored at 450 nm by a microplate reader (FLUOstar Omega (BMG, Labtech, Offenburg, Germany) in kinetic mode for 30 min. Catalytic activity in mU/ml had been used to express the results (Hu et al, 2019). Three independent experiments were performed.

2.10. Assessment of Citrate Synthase (CS) Activity

The investigation of activity of CS, the enzyme which catalyzes the synthesis of citrate from oxaloacetate in Krebs cycle, was performed by CS activity kit, (MAK193, Sigma-Aldrich Chemie GmbH, Germany), using 20 μ l sample lysate/group. To quantify CS activity, the assay measures the color development of TNB chromophore (2-nitro-5-thiobenzoic acid), produced from Ellman's Reagent, 5,5'-Dithiobis-2-Nitrobenzoic Acid (DTNB) and the CoA-SH generated during the citrate synthesis. The colorimetric product is directly proportional to the amount of active enzyme present in the samples (Srere, 1969; Petrohai et al, 2005). The absorbance measurements at 412 nm were performed using a microplate reader (FLUOstar Omega (BMG, Labtech, Offenburg, Germany) in kinetic mode for 30 min. Each step was done according to the provider's instructions. The enzyme's activity was reported as mU/mL. Each sample was tested three times.

2.11. Western Blot Analysis

To assess the effect of the SIM on the expression of several critical protein players involved in the energy metabolism, western blot analysis was performed as described previously (Rauca et al, 2018; Rauca et al, 2021). Thus, to determine HIF-1 α levels in cell lysates, 80 μ g of total proteins from each lysate were loaded/per lane onto a 7.5% polyacrylamide gel. To reveal the expression of SCD1, 60 μ g of total protein were used in each group, respectively 50 μ g of total protein for acyl-Coenzyme A oxidase 3 (ACOX3) and mitochondrially encoded NADH dehydrogenase 1 (mt-ND1). To quantify FASN, we used 40 μ g of total protein, while for facilitated glucose transporters, solute carrier family 2 member 1 (SLC2a1) / glucose transporter 1 (GLUT1), total protein kinase B (t-Akt) also known as serine/threonine-specific protein kinases and phosphorylated Akt (pAkt) 30 μ g of total protein lysates were needed. To detect the levels of these proteins 10% polyacrylamide gel was used. To block membranes, 5% skimmed milk powder dissolved in TBS-T (0.1% Tween) was used for 1 h at room temperature (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the membranes were immunoblotted overnight at 4°C against the primary antibodies as follows, rabbit monoclonal IgG anti-mouse HIF-1 α diluted 1:500 (ab179483, Abcam, Cambridge, United Kingdom), rabbit monoclonal IgG anti-mouse SCD1 1:500 (2794, Cell Signaling, United States), rabbit polyclonal IgG anti-mouse ACOX3 1:1000 dilution (PA5-22373, Pierce, Thermo Fisher Scientific, Inc.), rabbit polyclonal IgG anti-mouse mt-ND1 1:1000 (SAB5700797, Sigma-Aldrich Chemie GmbH, Germany), mouse monoclonal IgG anti-mouse FASN 1:1000 (sc-48357, Santa Cruz Biotechnology, Inc., United States), rabbit polyclonal IgG anti-mouse GLUT1 1:1000 (SAB4502803, Sigma-Aldrich Chemie GmbH, Germany), rabbit monoclonal IgG anti-mouse t-Akt 1:1000 (4685, Cell Signaling, United States), rabbit

polyclonal IgG anti-mouse p-Akt 1:1000 (sc-7985, Santa Cruz Biotechnology, Inc., United States). As primary antibody to test loading control, β -actin 1:1000 diluted rabbit polyclonal IgG anti-mouse β -actin (A2103, Merck, Darmstadt, GER) was used. As secondary antibodies, a goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG (sc-2005, Santa Cruz Biotechnology, Inc., United States) was utilized for FASN detection, while goat anti-rabbit IgG HRP-conjugated was used to quantify HIF-1 α , SCD1, ACOX3, mt-ND1, GLUT1, t-Akt and p-Akt (sc-2004, Santa Cruz Biotechnology, Inc., United States). Each secondary antibody was diluted 1:2500 in TBS-T and the membranes were incubated for 1 h at room temperature. Moreover, membranes were washed in TBS-T three times for 5 minutes before and after the incubation with the secondary antibody (Rauca et al, 2018; Rauca et al, 2021). Data from at least three independent experiments were presented as mean $\pm$ SD and each protein's level of expression was compared to control group expression.

2.12. Statistical Analysis

Mean $\pm$ SD was used to report data obtained from different experiments. Unpaired Student's t-test was used to statistically compare the effects of SIM-treatment on different enzymes activities / levels with those from untreated cell lysates. GraphPad Prism version 7 for Windows (San Diego, CA, USA) was used to conduct all statistical analyses. A *p*-value lower than 0.05 was defined as significant.

3. Results

SIM hindered key regulatory molecules of cancer cell metabolism reprogramming

Based on our previous data that showed that 3.5 μ M SIM induced inhibition of B16.F10 murine melanoma cell proliferation by 50% under normoxia (Alupe et al, 2014), we assessed the effects of this lipophilic statin on the production of two regulatory proteins for cancer cell aggressiveness and metabolism reprogramming: pAkt (Cheung et al, 2008; Stahl et al, 2004; Davies et al, 2009; Kwong and Davies, 2013; McGrail et al, 2022) and HIF-1 (Lartigau et al, 1997; Kuphal et al, 2010; Mouriaux et al, 2014; Koch et al, 2015; Stine et al, 2015). Therefore, mRNA expression was assessed *via* RNA-seq analysis, RT-qPCR and protein levels *via* western blot analysis. Our data suggested no changes in the mRNA levels corresponding to both regulators, in the SIM-treated group compared to the untreated group (Supplementary Table 1. All genes; Figure 1). Nevertheless, SIM exerted suppressive effects on protein production for the subunit α of HIF-1 (HIF-1 α) by 30% (*p*<0.01) (Figure 3 A,B), and significantly reduced the ratio of the active form of Akt/total Akt production by 1.4 fold (*p*<0.001) (Figure 3 C,D) in comparison with the control protein levels. These data suggested a regulatory mechanism modulated by SIM at the translational level. In tight connection with this finding, significant changes were identified in the mRNA profile for proteins involved in postsynthetic modification of both Akt, such as: heat shock protein 90 (HSP90) (300 times reduction after treatment compared to control, *p*<0.05), and PTEN induced putative kinase 1 (PINK1) (by almost 2 times, *p*<0.05) (Taipale and Jarosz, 2010; Junttila and Westermarck, 2008; Benitez, et al, 2017), as well as in HIF-1 α translation: eukaryotic translation initiation factor 4A1 (eIF4A1) (FR=-17.75, *p*<0.05), ribosomal protein S6 kinase polypeptide 2 (Rps6kb2) (FR=-3, *p*<0.05) and ribosomal protein S6 (RPS6) (FR=-1.6, *p*<0.05) (Semenza, 2012; Zhang et al, 2014; Lehman et al, 2022) (Table 2; Figure 4).

Table 2. DEG in Akt and HIF-1 translation.

Transcript_ID	Gene_ID	FR	p-value	FDR-adjusted p-value	Involvement of factors	Gene_name	Gene_description
ENSMUST00000004044	ENSMUSG00000026098	1.66	0.000	0.033	Akt translation and postsynthetic modification	PIK3R3	phosphoinositide 3-kinase regulatory subunit 3 [Source: MGI Symbol; Acc: MGI:109277]
ENSMUST00000005056	ENSMUSG00000009276	1.80	0.001	0.048	Akt translation and postsynthetic modification	PTEN1	PTEN induced putative kinase 1 [Source: MGI Symbol; Acc: MGI:191619]
TC025_00006060	ENSMUSG000000021270	-328.82	0.001	0.043	Akt translation and postsynthetic modification	HSP90AA1	heat shock protein 90, alpha (cytosolic), class A member 1 [Source: MGI Symbol; Acc: MGI:106250]
TC025_00006181	ENSMUSG000000017843	1.71	0.021	0.165	Akt translation and postsynthetic modification	PPP2R5C	protein phosphatase 2, regulatory subunit B, gamma [Source: MGI Symbol; Acc: MGI:1349475]
ENSMUST00000007609	ENSMUSG000000090360	1.87	0.014	0.113	HIF-1 α translation and postsynthetic modification	MLP3K3	mitogen-activated protein kinase 3 [Source: MGI Symbol; Acc: MGI:1348496]
ENSMUST00000009343	ENSMUSG000000020190	1.66	0.006	0.090	HIF-1 α translation and postsynthetic modification	MKNK2	MAP kinase-interacting serine/threonine kinase 2 [Source: MGI Symbol; Acc: MGI:894279]
ENSMUST00000012151	ENSMUSG000000093661	1.63	0.001	0.050	HIF-1 α translation and postsynthetic modification	EIF4E3	eukaryotic translation initiation factor 4E member 3 [Source: MGI Symbol; Acc: MGI:1914142]
ENSMUST00000012677	ENSMUSG000000095796	-17.75	0.002	0.059	HIF-1 α translation and postsynthetic modification	EIF4A1	eukaryotic translation initiation factor 4A1 [Source: MGI Symbol; Acc: MGI:85303]
ENSMUST00000025749	ENSMUSG000000024830	-3.00	0.015	0.139	HIF-1 α translation and postsynthetic modification	RPS6KB2	ribosomal protein S6 kinase, polypeptide 2 [Source: MGI Symbol; Acc: MGI:1927343]
ENSMUST00000010174	ENSMUSG000000028495	-1.60	0.017	0.147	HIF-1 α translation and postsynthetic modification	RPS6	ribosomal protein S6 [Source: MGI Symbol; Acc: MGI:98159]

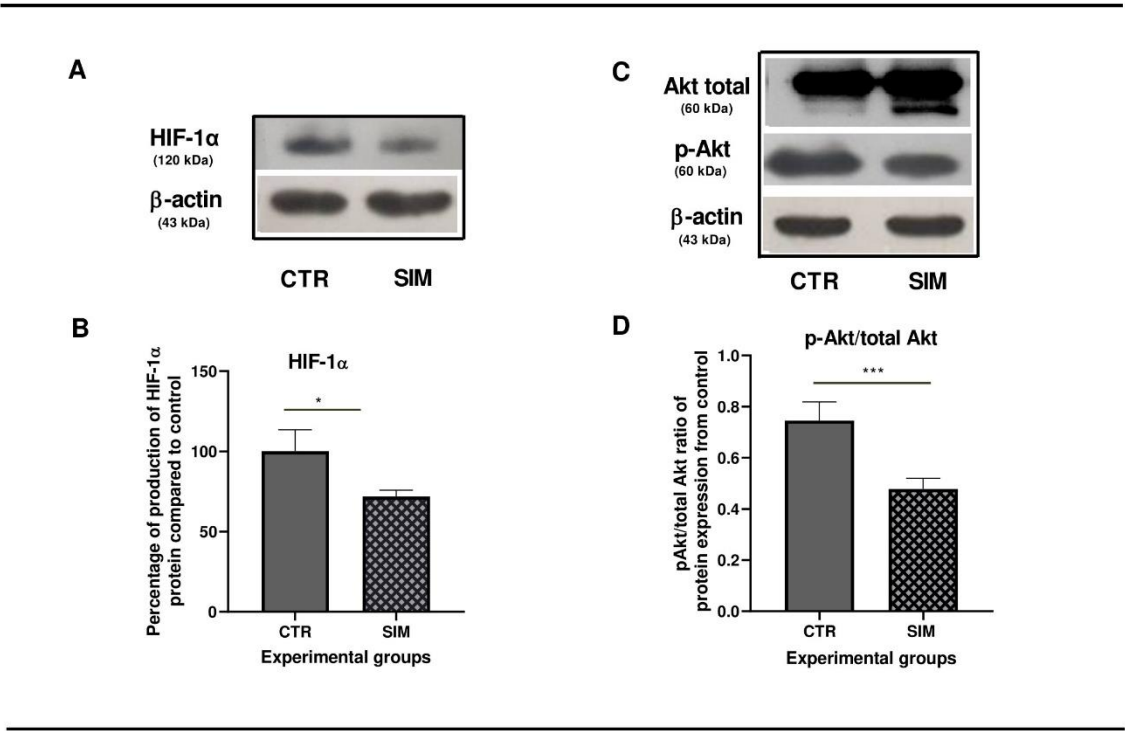


Figure 3. Protein levels of two regulatory molecules after 3.5 μM SIM-administration in B16.F10 cells under normoxia. (A, C) Western blot images of the expression levels of HIF-1α, total Akt and pAkt. β-actin was used as loading control; (B) Percentages of the HIF-1α levels in SIM-treated cells (SIM) in comparison with the production of the same protein in control lysates (CTR); (D) The ratio of expression levels of pAkt protein of total Akt levels in comparison with the production of the same protein in control lysates (CTR). Data were expressed as mean ± SD of at least three independent measurements; * $p < 0.05$; *** $p < 0.001$. **Abbreviations:** β-actin: beta-actin; CTR: control group; HIF-1α: hypoxia-inducible factor 1 alpha; pAkt: phosphorylated Akt; SIM: simvastatin treated group; total Akt: total Akt (protein kinase B).

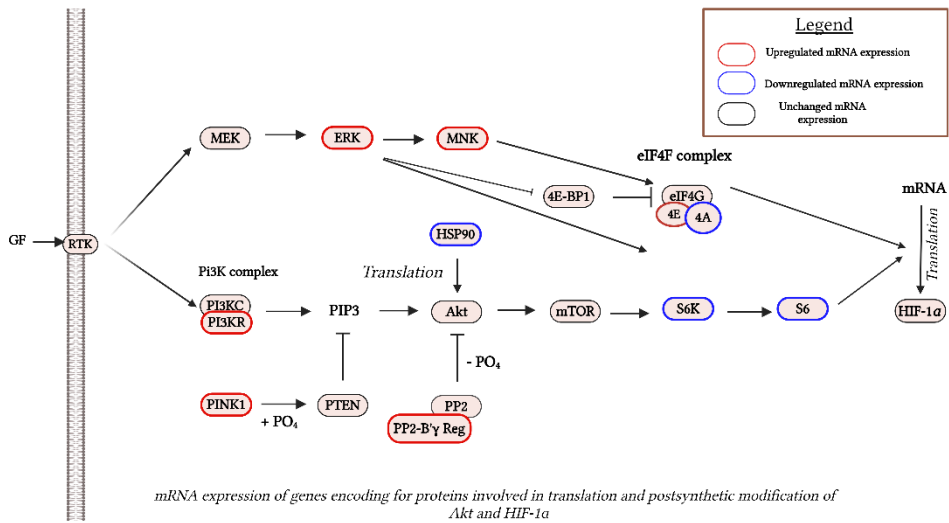


Figure 4. mRNA levels of genes encoding for proteins involved in translation and postsynthetic modification of Akt and HIF-1 proteins after SIM-administration. Red outlines indicate the up-regulated genes and green outlines indicate the down-regulated genes, revealed by RNA-Seq analysis. KEGG pathway maps served as sources for all reaction stages. Created in BioRender. Gabriela, G. (2024) BioRender.com/137i799. Abbreviations: 4E-BP1: eukaryotic initiation factor 4E-binding protein 1; Akt: protein kinase B; ERK: extracellular signal-regulated kinase; eIF4F: eukaryotic initiation factor 4F; GF: growth factor; HIF-1 α : hypoxia-inducible factor 1 alpha; HSP90: heat shock protein 90; mTOR: mechanistic target of rapamycin; MEK: MAPK/ERK kinase; MNK: MAPK-interacting kinase; PI3KC: phosphoinositide 3-kinase catalytic subunit; PI3KR: phosphoinositide 3-kinase regulatory subunit; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; PP2: protein phosphatase 2; PTEN: phosphatase and tensin homolog; PINK1: PTEN-induced kinase 1; PO4: phosphate group; PPR-B γ Reg: protein phosphatase 2, regulatory subunit B', gamma; RTK: receptor tyrosine kinase; S6: ribosomal protein S6; S6K: ribosomal protein S6 kinase.

3.1. SIM Treatment Altered Expression of mRNA for SREBP1 and FOXM1 - Two Important Regulators of Cholesterol Biosynthesis and Tumor Development

As statins are proven to be competitive inhibitors of cholesterol biosynthesis (Istvan and Deisenhofer, 2001), as well as due to the importance of this pathway for tumor development and progression (Silvente and Poirot, 2014; Kuzu et al, 2016), we explored the effects of SIM on the transcriptomic profile of the cholesterol biosynthesis and regulation. Our data indicated that two positive regulators of cholesterol biosynthesis, such as sterol regulatory element binding proteins (SREBP1) (Weber et al, 2004), as well as of forkhead box M1 (FOXM1) factor associated with mevalonate pathway regulation, tumorigenesis and glucose metabolism (Koo et al, 2012; Zhang et al, 2017; Wang et al, 2016), were significantly reduced by SIM (Table 3; Figure 5). Moreover, insulin-induced gene proteins 1 and 2 (INSIG-1 and INSIG-2), responsible for maintaining SRBEP in inactive form (Yang et al, 2002; Yabe et al, 2002), were both 2-fold overexpressed after the same treatment (Table 3; Figure 5). In addition to these findings, except for the gene encoding for the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMG-CR) (Supplementary Table 1. All genes), transcripts of genes encoding for most enzymes of the same pathway were upregulated in response to SIM-treatment, as follows: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMG-CS1, FR=3.48; $p<0.001$), squalene epoxidase (SQLE, FR=2.38; $p<0.05$), mevalonate (diphospho) decarboxylase (MVD, FR=2.7; $p<0.01$), isopentenyl-diphosphate isomerase (IDI1, FR=3.21; $p<0.01$), farnesyl diphosphate synthase (FDPS, FR=1.95; $p<0.01$), and acetyl-coenzyme A acetyl-transferase 2 (ACAT2, FR=3.25; $p<0.01$) (Table 3). These findings suggest that SIM-induced inhibition of key regulatory molecules was counteracted *via* up-regulation of genes encoding enzymes for isoprenoids production, which are important metabolites for postsynthetic modification of proteins which are involved in signaling pathways related to the melanoma cell proliferation (Bjarnadottir et al, 2015; Kimbung et al, 2016; Lazaro-Mixteco et al, 2022).

Table 3. DEG in cholesterol metabolism pathway.

Transcript_ID	Gene_ID	FR	p-value	FDR-adjusted p-value	gene_name	Gene_description
ENSMUST00000134660	ENSMUSG00000020538	-1.52	0.004	0.077	SREBF1	sterol regulatory element binding transcription factor 1 [Source:MGI Symbol;Acc:MGI:107606]
ENSMUST00000059155	ENSMUSG00000045294	2.18	0.002	0.056	INSIG1	insulin induced gene 1 [Source:MGI Symbol;Acc:MGI:1916289]
ENSMUST00000038118	ENSMUSG00000003721	1.60	0.000	0.038	INSIG2	insulin induced gene 2 [Source:MGI Symbol;Acc:MGI:1920249]
ENSMUST00000161818	ENSMUSG00000003721	1.75	0.022	0.166	INSIG2	insulin induced gene 2 [Source:MGI Symbol;Acc:MGI:1920249]
ENSMUST00000073316	ENSMUSG000000001517	-1.87	0.023	0.169	FOXM1	forkhead box M1 [Source:MGI Symbol;Acc:MGI:1347487]
TCONS_00007283	ENSMUSG000000093930	3.48	0.000	0.041	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 [Source:MGI Symbol;Acc:MGI:107592]
ENSMUST00000022977	ENSMUSG00000022351	2.39	0.012	0.122	SQLE	squalene epoxidase [Source:MGI Symbol;Acc:MGI:109296]
ENSMUST00000006692	ENSMUSG000000006517	2.71	0.004	0.076	MVD	mevalonate (diphospho) decarboxylase [Source:MGI Symbol;Acc:MGI:2179327]
ENSMUST00000169314	ENSMUSG000000058258	3.21	0.003	0.072	IDI1	isopentenyl-diphosphate delta isomerase [Source:MGI Symbol;Acc:MGI:2442264]
ENSMUST00000081848	ENSMUSG000000059743	1.95	0.002	0.057	FDPS	farnesyl diphosphate synthetase [Source:MGI Symbol;Acc:MGI:104888]
TCONS_00011825	ENSMUSG00000023832	3.25	0.002	0.056	ACAT2	acetyl-Coenzyme A acetyltransferase 2 [Source:MGI Symbol;Acc:MGI:87871]
ENSMUST00000162848	ENSMUSG000000033105	3.50	0.021	0.162	LSS	lanosterol synthase [Source:MGI Symbol;Acc:MGI:1336155]
ENSMUST00000047973	ENSMUSG000000034926	2.66	0.001	0.043	DHCR24	24-dehydrocholesterol reductase [Source:MGI Symbol;Acc:MGI:1922004]
ENSMUST00000052725	ENSMUSG000000032018	2.26	0.000	0.042	SCSD	sterol-C5-desaturase [Source:MGI Symbol;Acc:MGI:1353611]
ENSMUST00000033715	ENSMUSG000000031349	2.80	0.002	0.053	NSDHL	NAD(P) dependent steroid dehydrogenase-like [Source:MGI Symbol;Acc:MGI:1099438]
ENSMUST00000025713	ENSMUSG00000024799	3.47	0.008	0.104	TM7SF2	transmembrane 7 superfamily member 2 [Source:MGI Symbol;Acc:MGI:1920416]
ENSMUST00000001507	ENSMUSG000000001467	2.03	0.002	0.055	CYP51	cytochrome P450, family 51 [Source:MGI Symbol;Acc:MGI:106040]

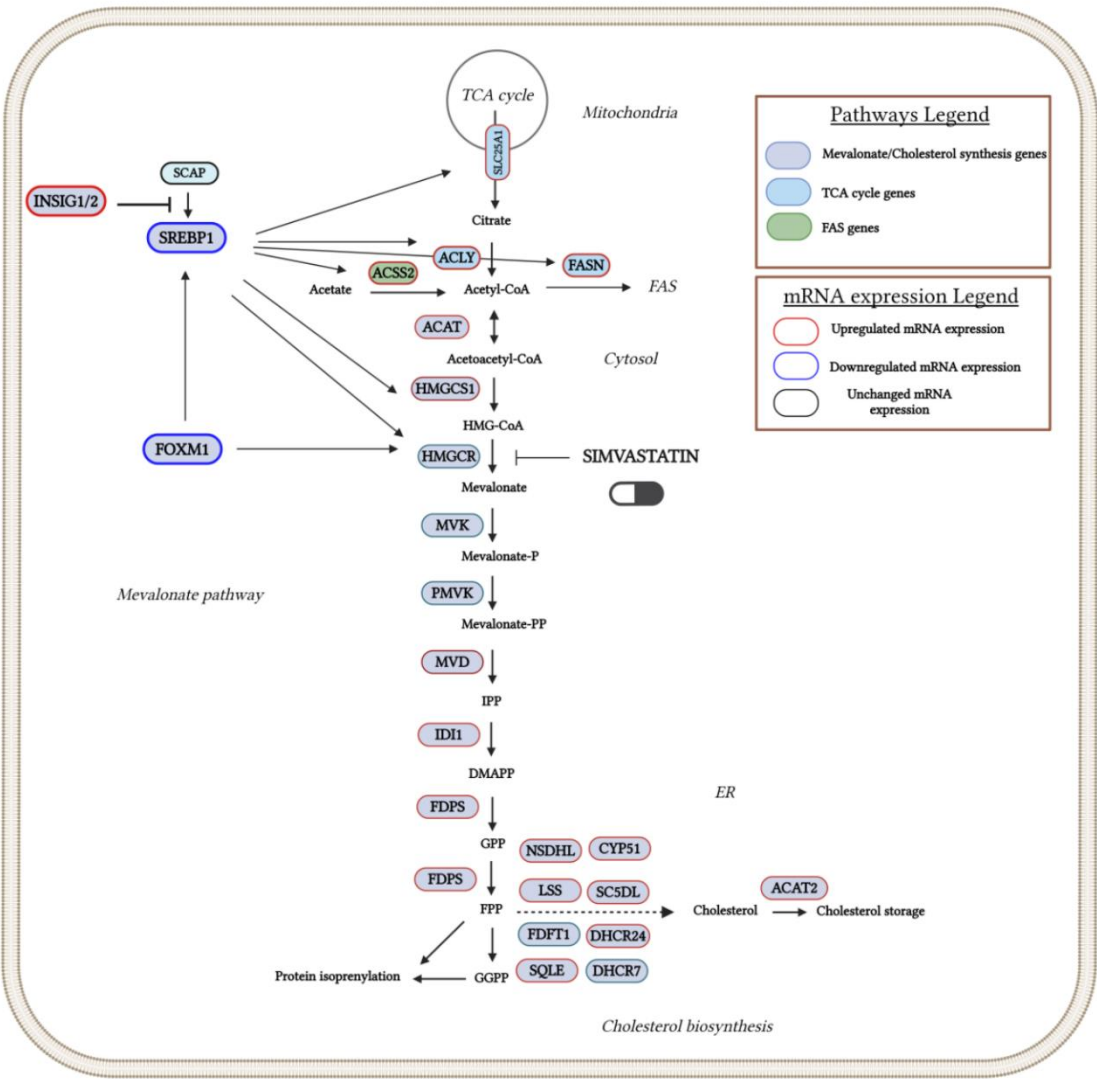


Figure 5. Overview of the cholesterol metabolism pathway and its regulation by SREBP1 and FOXM1 regulatory factors in SIM-treated melanoma cells. Red outlines indicate the up-regulated genes and green outlines indicate the down-regulated genes which were revealed by RNA-Seq analysis. KEGG pathway maps served as sources for all reaction stages. Created in BioRender. Gabriela, G. (2024) BioRender.com/g44x434. Abbreviations: ACAT: acetyl-CoA acetyltransferase; ACAT2: acetyl-CoA acetyltransferase 2; ACLY: ATP citrate lyase; ACSS2: acyl-CoA synthetase short-chain family member 2; CYP51: cytochrome P450 family 51; DHCR24: 24-dehydrocholesterol reductase; DHCR7: 7-dehydrocholesterol reductase; FDPS: farnesyl diphosphate synthase; FAS: fatty acid synthase; FASN: fatty acid synthase; FDFT1: farnesyl-diphosphate farnesyltransferase 1 (squalene synthase); FOXM1: forkhead box M1; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; INSIG1/2: insulin induced gene 1/2; IDI1: isopentenyl-diphosphate delta isomerase 1; LSS: lanosterol synthase; MVD: mevalonate diphosphate decarboxylase; MVK: mevalonate kinase; NSDHL: NAD(P) dependent steroid dehydrogenase-like; PMVK: phosphomevalonate kinase; SC5DL: sterol-C5-desaturase-like; SCAP: SREBP cleavage-activating protein; SREBP1: sterol regulatory element-binding protein 1; SQLE: squalene epoxidase.

3.2. SIM Effects on Transcriptomic Profile of Metabolism of B16.F10 Melanoma Cells Under Normoxia

As SIM affected the protein synthesis of two metabolic reprogramming regulators HIF-1 α and Akt, we investigated by RNA-seq the mRNA levels of genes encoding for proteins involved in several metabolic pathways regulated by these factors. To validate this data, RT-qPCR was used to assess the expression of HIF-1 α , Akt and direct/indirect targets in the metabolic pathways analysed (**Figure 1**). We found full concordance between RT-qPCR data and RNA-Seq results in terms of both the amount and the direction of expression changes. For FASN, isocitrate dehydrogenase 1 (IDH1) and solute carrier family 1 glutamate transporter member 4 (SLC1A4) genes, the expression levels were slightly higher than those obtained by RNA-Seq, while the expression of all the others was comparable between the two techniques.

Our data revealed a panel of 1513 out of 15905 genes with at least 1.5 FR in expression after SIM treatment, with 817 transcripts upregulated, and 696 downregulated compared to the control group (**Supplementary Table 1. All genes**). Functional enrichment analysis showed that SIM treatment favored the transcription of genes encoding proteins which are involved in glucose, glutamine and fatty acids transport, glycolysis, PPP, TCA cycle, OXPHOS, fatty acids synthesis (FAS), fatty acids elongation (FAE), peroxisomal β -oxidation (FAO) and suppressed transcripts related to ribonucleotide synthesis, OXPHOS and mitochondrial biogenesis as showed by the clustering of DEG in the hierarchical representation (**Figures 6 and 7; Table 4**). Namely, SIM treatment doubled the mRNA levels of genes encoding for glucose metabolic pathways such as glycolysis: aldolase C (Aldoc) (FR=2.06; $p=0.0027$), enolase 2 (Eno2) (FR=1.96; $p=0.0248$) and facilitated glucose transporter solute carrier family 2 member 6 (SLC2A6 or GLUT6) (FR=2.5-fold increase; $p=0.0108$) and PPP: G6PD (FR=1.5; $p=0.0003$) and 6-phosphogluconolactonase (PGLS) (FR=1.87; $p=0.0305$) compared to the control levels (**Table 4; Figure 6 and 7**). Although there were increased mRNA levels of genes encoding for proteins involved in mitochondrial metabolism, such as IDH1 and several electron transport chain (ETC) subunits, one subunit of the ETC complex I, NADH dehydrogenase: Ubiquinone Oxidoreductase Subunit B10 (Ndufb10) was downregulated (FR=-1.53; $p=0.0294$) and the mitochondrial biogenesis was affected by SIM treatment (nuclear respiratory factor 1 (NRF1) downregulation; $p<0.05$) (**Table 4; Figure 7 and 8**). Moreover, our data suggested that SIM administration favored both lipolysis and FAS as increased levels of transcripts were noted for lipolysis enzymes: adipose triglyceride lipase (ATGL) by twofold (FR=1.92; $p<0.05$), monoacylglycerol lipase (MAGL) by ninefold (FR=9.18; $p<0.05$), phospholipase D1 (PLD1) by fivefold (FR=5.28; $p=0.017$), and for FAS and FAE: solute carrier family 25 member 1 (SLC25A1) (FR=1.96; $p=0.001$) transporter of mitochondrial citrate into the cytosol (**Hlouschek et al, 2018**) and ATP citrate lyase (ACLY), the enzyme responsible for linking of TCA to cytosolic FAS (FR=1.65; $p=0.0037$), FASN (FR=1.98; $p=0.002$) and elongation of long chain fatty acids family, member 6 (ELOVL6; FR=2.02; $p=0.004$) (**Table 4; Figure 6 and 7**). Also, SIM favored UFAs abundance by upregulating SCD1 mRNA expression by twofold (FR=1.86; $p=0.005$) (**Table 4; Figure 6 and 7**). Interestingly, SIM did not significantly influenced mitochondrial FAO (**Supplementary Table 1. All genes**), but upregulated mRNA level of ACOX3, the rate-limiting enzyme of the peroxisomal FAO (FR=2.13; $p=0.0065$) (**Table 4; Figure 6 and 7**). All these data linked with the upregulation of mRNA level for prostaglandin D2 synthase (PGDS) (FR=2.81; $p=0.001$), suggested that SIM modulated an increase in prostaglandin synthesis in melanoma cells (**Table 4; Figure 6 and 7**). Our transcriptomics findings indicated that excepting for glutamine related ribonucleotide synthesis, SIM enhanced glutamine utilization, implied by increased expression of glutamine transporters or enzymes such as SLC1A4 (FR=1.55; $p=0.0041$), alanyl aminopeptidase (ANPEP) (FR=4.69; $p=0.0214$) and glutamine fructose-6-phosphate transaminase 1 (GFPT1) (FR=1.5; $p=0.0043$) (**Table 4; Figure 7**). Because glutamine metabolism can replenish TCA cycle intermediates, it provides a carbon and nitrogen source for energy, nucleotide and lipid synthesis, promoting cell proliferation (**Kovacević and Morris, 1972; Mohamed et al, 2014; Zacharias et al, 2017; Demas et al, 2019; Gaglio et al, 2011; Kerr et al, 2016**). Altogether, our RNA-seq data suggested that SIM treatment enhanced cell metabolism at the mRNA level, mainly by upregulating mRNA expression of genes encoding for: transporters of glucose, glutamine and FAs; glucose metabolism; mitochondrial oxidative metabolism and lipid metabolism in B16.F10 melanoma cells.

Table 4. DEG in metabolism.

Transcript_ID	Gene_ID	FR	p-value	FDR-adjusted p-value	Pathway	Gene_name	Gene_description
ENSMUTST0000017534	ENSMUG00000007390	2.06	0.003	0.064	Glycolysis	ALDOC	aldolase C, fructose biphosphate [Source:MGI Symbol;Acc:MGI:161863]
ENSMUTST0000012436	ENSMUG00000004267	1.97	0.023	0.177	Glycolysis	ENO2	enolase 2, gamma isoform [Source:MGI Symbol;Acc:MGI:953194]
ENSMUTST0000044782	ENSMUG00000000967	2.57	0.011	0.117	Glycolysis	SLC2A6	solute carrier family 2 (facilitated glucose transporter), member 6 [Source:MGI Symbol;Acc:MGI:2443186]
ENSMUTST0000014341	ENSMUG00000001307	1.87	0.036	0.197	PPP	PGLS	9-phosphophenylacetate [Source:MGI Symbol;Acc:MGI:1913421]
ENSMUTST0000004327	ENSMUG00000003140	1.50	0.006	0.038	PPP	G6PD	glucose 6-phosphate dehydrogenase [Source:MGI Symbol;Acc:MGI:109979]
ENSMUTST0000007739	ENSMUG00000002559	1.72	0.007	0.092	TCA cycle	IDH1	isocitrate dehydrogenase 1 (NADP+), soluble [Source:MGI Symbol;Acc:MGI:98413]
ENSMUTST0000003622	ENSMUG00000003238	1.86	0.001	0.147	TCA cycle	SLC25A1	solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 [Source:MGI Symbol;Acc:MGI:1345283]
ENSMUTST0000016111	ENSMUG00000002917	1.65	0.004	0.075	TCA cycle	ACLY	ATP citrate lyase [Source:MGI Symbol;Acc:MGI:10251]
ENSMUTST0000004942	ENSMUG00000002169	1.59	0.026	0.192	Glutamine metabolism	SLC38A1	solute carrier family 38, member 1 [Source:MGI Symbol;Acc:MGI:145905]
ENSMUTST0000023999	ENSMUG00000002362	1.44	0.007	0.096	Glutamine metabolism	SLC38A2	solute carrier family 38, member 2 [Source:MGI Symbol;Acc:MGI:1918010]
ENSMUTST0000004634	ENSMUG00000002042	1.55	0.004	0.077	Glutamine metabolism	SLC1A4	solute carrier family 1 (glutamate/neurotransmitter amino acid transporter), member 4 [Source:MGI Symbol;Acc:MGI:2135601]
ENSMUTST0000013283	ENSMUG00000000978	1.74	0.008	0.091	Glutamine metabolism	RRM1	ribonucleotide reductase M1 [Source:MGI Symbol;Acc:MGI:99180]
ENSMUTST0000014759	ENSMUG00000002649	2.23	0.012	0.121	Glutamine metabolism	RRM2	ribonucleotide reductase M2 [Source:MGI Symbol;Acc:MGI:99181]
ENSMUTST0000001715	ENSMUG00000000165	1.54	0.014	0.134	Glutamine metabolism	GSTT3	glutathione S-transferase, theta 3 [Source:MGI Symbol;Acc:MGI:2143526]
ENSMUTST0000004904	ENSMUG00000000962	4.70	0.021	0.165	Glutamine metabolism	ANPEP	diacyl (membrane) aminopeptidase [Source:MGI Symbol;Acc:MGI:300846]
ENSMUTST0000013658	ENSMUG00000000902	1.50	0.004	0.077	Glutamine metabolism	GPTT1	glutamate, pyruvate, oxaloacetate transaminase 1 [Source:MGI Symbol;Acc:MGI:15968]
ENSMUTST0000002392	ENSMUG00000004514	1.73	0.010	0.114	OXPHOS	mt-ND1	mitochondrially encoded NADH dehydrogenase 1 [Source:MGI Symbol;Acc:MGI:101787]
ENSMUTST0000002396	ENSMUG00000004455	1.68	0.005	0.083	OXPHOS	mt-ND2	mitochondrially encoded NADH dehydrogenase 2 [Source:MGI Symbol;Acc:MGI:102500]
ENSMUTST0000002414	ENSMUG00000004353	1.96	0.003	0.064	OXPHOS	mt-ND4	mitochondrially encoded NADH dehydrogenase 4 [Source:MGI Symbol;Acc:MGI:102498]
ENSMUTST0000004013	ENSMUG00000000787	2.12	0.007	0.091	OXPHOS	mt-ND4L	mitochondrially encoded NADH dehydrogenase 4L [Source:MGI Symbol;Acc:MGI:102497]
ENSMUTST00000144285	ENSMUG00000000008	1.33	0.029	0.193	OXPHOS	Nadh10	NADH ubiquinone oxidoreductase subunit B10 [Source:MGI Symbol;Acc:MGI:1915502]
ENSMUTST0000002421	ENSMUG00000004779	2.05	0.008	0.089	OXPHOS	mt-CYTB	mitochondrially encoded cytochrome b [Source:MGI Symbol;Acc:MGI:102501]
ENSMUTST00000028781	ENSMUG00000000571	4.00	0.051	0.196	OXPHOS	CONAH1	cytochrome c oxidase, subunit 6B1 [Source:MGI Symbol;Acc:MGI:107560]
ENSMUTST0000002405	ENSMUG000000004354	1.79	0.026	0.182	OXPHOS	mt-COX2	mitochondrially encoded cytochrome c oxidase II [Source:MGI Symbol;Acc:MGI:102503]
ENSMUTST0000002407	ENSMUG00000004356	1.55	0.022	0.168	OXPHOS	mt-ATP8	mitochondrially encoded ATP synthase 8 [Source:MGI Symbol;Acc:MGI:98928]
ENSMUTST0000013266	ENSMUG00000000948	-1.36	0.009	0.104	OXPHOS	SRF1	nuclear respiratory factor 1 [Source:MGI Symbol;Acc:MGI:133235]
ENSMUTST0000000949	ENSMUG000000002174	9.18	0.002	0.062	Lipolysis	MAGL	monoacylglycerol lipase (monoacylglycerol lipase) [Source:MGI Symbol;Acc:MGI:134002]
ENSMUTST0000014416	ENSMUG00000002599	1.62	0.002	0.061	Lipolysis	ATGL	adipose triglyceride lipase (panin-1-like phospholipase domain containing 2) [Source:MGI Symbol;Acc:MGI:191410]
ENSMUTST0000005555	ENSMUG00000002153	1.99	0.002	0.053	FAS	FASN	fatty acid synthase [Source:MGI Symbol;Acc:MGI:91482]
ENSMUTST0000007402	ENSMUG000000004128	2.02	0.004	0.076	FAS	ELOVL6	ELOVL family member 6, elongation of long chain fatty acids (xanth)
ENSMUTST0000002257	ENSMUG00000002109	2.40	0.003	0.068	FAS	ELOVL7	ELOVL family member 7, elongation of long chain fatty acids (xanth)
ENSMUTST0000001156	ENSMUG00000001378	1.52	0.021	0.165	FAS	HCCD2	3-hydroxyacyl CoA dehydratase 2 [Source:MGI Symbol;Acc:MGI:191807]
ENSMUTST0000000065	ENSMUG00000000918	1.68	0.006	0.019	FAS	SLC27A1	solute carrier family 27 (fatty acid transporter), member 1 [Source:MGI Symbol;Acc:MGI:134737]
ENSMUTST0000001331	ENSMUG000000017071	1.86	0.005	0.083	FAS	SCD1	stearoyl-Coenzyme A desaturase 1 [Source:MGI Symbol;Acc:MGI:98259]
ENSMUTST00000029135	ENSMUG000000027005	1.87	0.006	0.022	FAD	ACSS2	acyl-CoA synthetase short-chain family, member 2 [Source:MGI Symbol;Acc:MGI:190810]
ENSMUTST0000000847	ENSMUG00000002908	2.11	0.006	0.093	FAD	ACOX3	acyl-Coenzyme A oxidase 3, peroxisomal [Source:MGI Symbol;Acc:MGI:1913156]
ENSMUTST0000012359	ENSMUG000000027057	5.28	0.017	0.148	Prostaglandin metabolism	PLD1	phospholipase D1 [Source:MGI Symbol;Acc:MGI:109351]
ENSMUTST0000014816	ENSMUG00000001509	2.41	0.001	0.042	Prostaglandin metabolism	PGC8	prostaglandin G/H synthase (cyclooxygenase) [Source:MGI Symbol;Acc:MGI:99281]

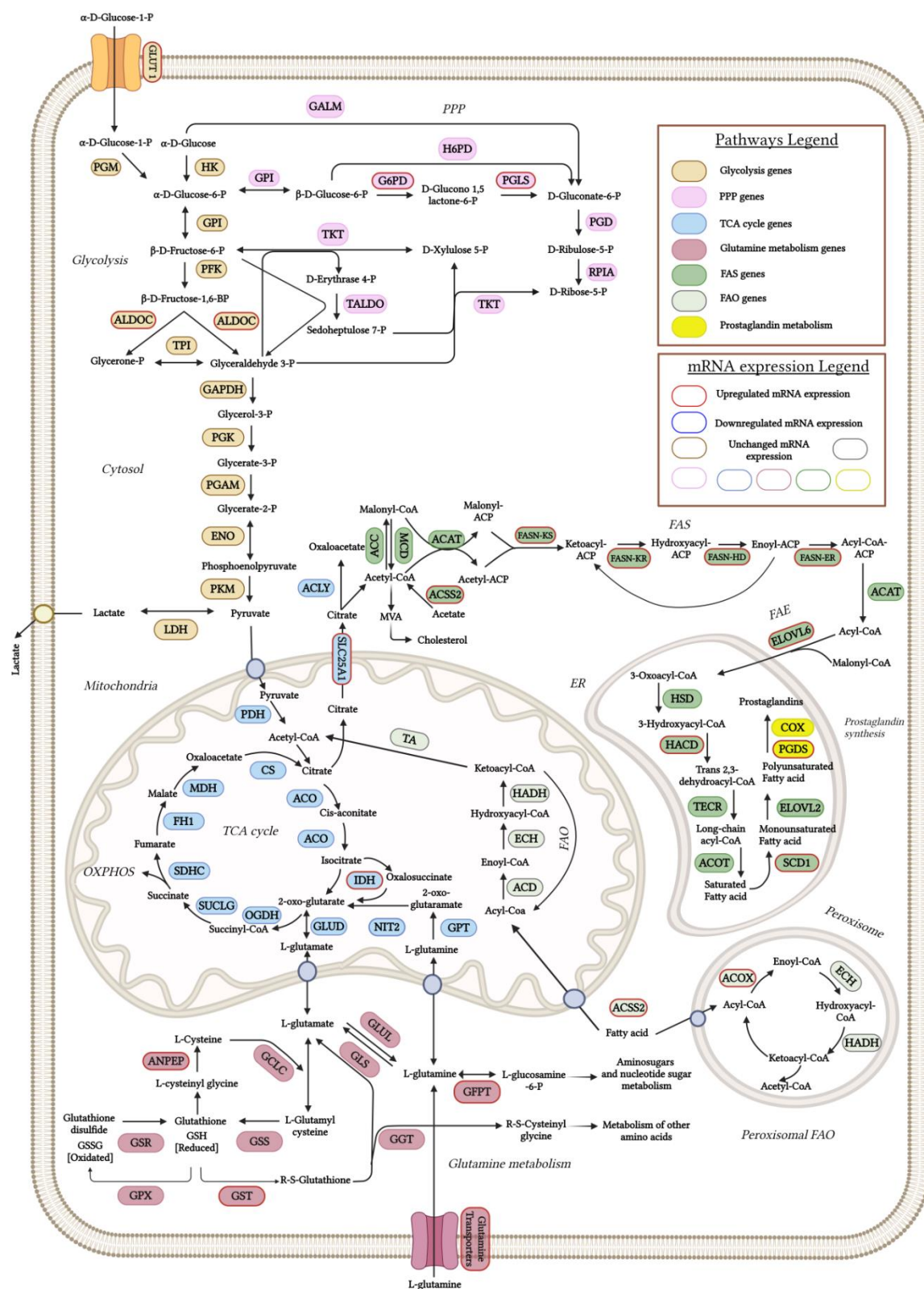


Figure 6. Schematic overview of the effects of SIM on the expression of genes encoding for proteins involved in metabolic pathways in B16.F10 murine melanoma cells. Red outlines indicate the up-regulated genes and green outlines indicate the down-regulated genes which were revealed by RNA-

Seq analysis. KEGG pathway maps served as sources for all reaction stages. Created in BioRender. Gabriela, G. (2024) BioRender.com/h93z324. Abbreviations: ACAT: acetyl-CoA acetyltransferase; ACLY: ATP citrate lyase; ACOX: acyl-CoA oxidase; ACS2: acyl-CoA synthetase short-chain family member 2; ACC: acetyl-CoA carboxylase; ACOT: acyl-CoA thioesterase; ANPEP: alanyl aminopeptidase; CS: citrate synthase; COX: cytochrome c oxidase; ELOVL2: elongation of very long chain fatty acids protein 2; ELOVL6: elongation of very long chain fatty acids protein 6; ENO: enolase; FAD: flavin adenine dinucleotide; FASN-KS: fatty acid synthase keto synthase; FASN-KR: fatty acid synthase keto reductase; FASN-HD: fatty acid synthase hydroxydecarboxylase; FASN-ET: fatty acid synthase enoyl reductase; FAO: fatty acid oxidation; FAS: fatty acid synthase; FH1: fumarate hydratase 1; GALM: galactose-1-phosphate mutase; GCLC: gamma-glutamylcysteine ligase catalytic subunit; GFPT: glutamine-fructose-6-phosphate transaminase; GCLC: gamma-glutamylcysteine synthetase; GLUL: glutamine synthetase; GLUD: glutamate dehydrogenase; G6PD: glucose-6-phosphate dehydrogenase; GPT: glutamate-pyruvate transaminase; GPI: glucose-6-phosphate isomerase; GSR: glutathione reductase; GSS: glutathione synthetase; GST: glutathione S-transferase; HACD: 3-hydroxyacyl-CoA dehydratase; HADH: hydroxyacid dehydrogenase; H6PD: hexose-6-phosphate dehydrogenase; HK: hexokinase; HSD: hydroxysteroid dehydrogenase; IDH: isocitrate dehydrogenase; MCD: malonyl-CoA decarboxylase; MDH: malate dehydrogenase; NIT2: nitrite reductase; OGDH: 2-oxoglutarate dehydrogenase; PDH: pyruvate dehydrogenase; PFK: phosphofructokinase; PGD: 6-phosphogluconate dehydrogenase; PGDS: prostaglandin D2 synthase; PGK: phosphoglycerate kinase; PGK: phosphoglycerate kinase; PGLS: 6-phosphogluconolactonase; PGM: phosphoglucomutase; PGAM: phosphoglycerate mutase; PKM: pyruvate kinase M; PPP: pentose phosphate pathway; RPIA: ribose-5-phosphate isomerase A; SCD1: stearoyl-CoA desaturase 1; SCO: sterol-14 α -demethylase; SDHC: succinate dehydrogenase cytochrome b subunit; SLC25A1: solute carrier family 25 member 1; SUCLG: succinate-CoA ligase gamma subunit; TAC: tricarboxylic acid cycle; TA: transaldolase; TALDO: transaldolase; TKT: transketolase; TGAPDH: 3-phosphoglycerate dehydrogenase; TNK: tyrosine kinase; TECR: trans-2-enoyl-CoA reductase.

3.3. Investigation of the SIM Effects the Metabolism of B16.F10 Melanoma Cells at Protein Level

To compare transcriptomics data results with the effects of SIM at protein level, we checked several proteins with a key role in cancer cell metabolism for their catalytic activity, through enzymatic assays (**Figure 9**) and protein production (**Figure 10**) *via* western blot analysis. Firstly, to assess if SIM treatment had the ability to modulate the glucose metabolism, we investigated whether the uptake of glucose through one isoform of the family of facilitated glucose transporters, GLUT1 was affected by treatment. While SIM did not affect GLUT1 mRNA expression ($p>0.05$) (**Supplementary Table 1. All genes**), it doubled the protein levels (**Figure 10A,B**), suggesting an enhanced uptake of glucose in SIM-treated cells. Although our transcriptomics data suggested that SIM upregulated expression of genes involved in glycolysis (**Table 4**), the catalytic activities of key enzymes were reduced by the treatment by almost 30% ($p<0.0001$) for one of the rate-limiting enzymes of the pathway - PFK1 (**Figure 9A**), and by 25% for LDH (**Figure 9B**), indirectly suggesting reduced lactate production in SIM-treated group. Also, our transcriptomics data indicated upregulation of transcripts of genes in the PPP; however, the catalytic activity of G6PD, the rate-limiting enzyme, was decreased by 30% ($p<0.01$) (**Figure 9C**) indicating an overall reduced pathway activity. Hence, this alteration likely led to a decrease in the nicotinamide adenine dinucleotide phosphate (NADPH) synthesis, affecting both redox homeostasis and nucleotide synthesis in which PPP intermediates take part in. Despite transcriptomics information revealing no changes in expression of genes in TCA cycles, except IDH1 (**Supplementary Table 1. All genes**), there was a substantial threefold increase ($p<0.0001$) in the catalytic activity of CS (**Figure 9D**), the first rate-limiting enzyme, indicating a mild re-triggering of TCA cycle activity due to SIM administration compared to control. Although our transcriptomics data suggested that SIM upregulated expression of genes involved in OXPHOS and peroxisomal FAO, the protein expression of mt-ND1 was slightly reduced in SIM-treated cells compared to

untreated cells (Table 4; Figure 10A,C). Using transcriptomics data we found that at mRNA level, both saturated FAS main enzyme FASN, and SCD1 the enzyme responsible for converting saturated FAs (SFAs) to UFAs exhibited significant increase in expression in SIM group compared to the control (Table 4). The expression signature was also seen at the protein level, with a significant twofold increase for FASN (Table 4; Figure 10 A,D), and threefold increase for SCD1 (Figure 10A,E) compared to control. The ACOX3 protein expression, enzyme crucial for peroxisomal FAO, was similar in SIM compared to the control (Table 4; Figure 10A,F). The observed increase in lipid metabolism and the preference for UFAs production in treated cells suggested an expected raise in the availability of monounsaturated fatty acids (MUFAs), providing protective effects against lipid peroxidation and endoplasmic reticulum (ER) stress (Gonzalez et al, 2018) and of polyunsaturated fatty acids (PUFAs), potentially leading to enhanced prostaglandins synthesis with significant implications for cellular processes (Wang et al, 2015; Ferreira et al, 2017).

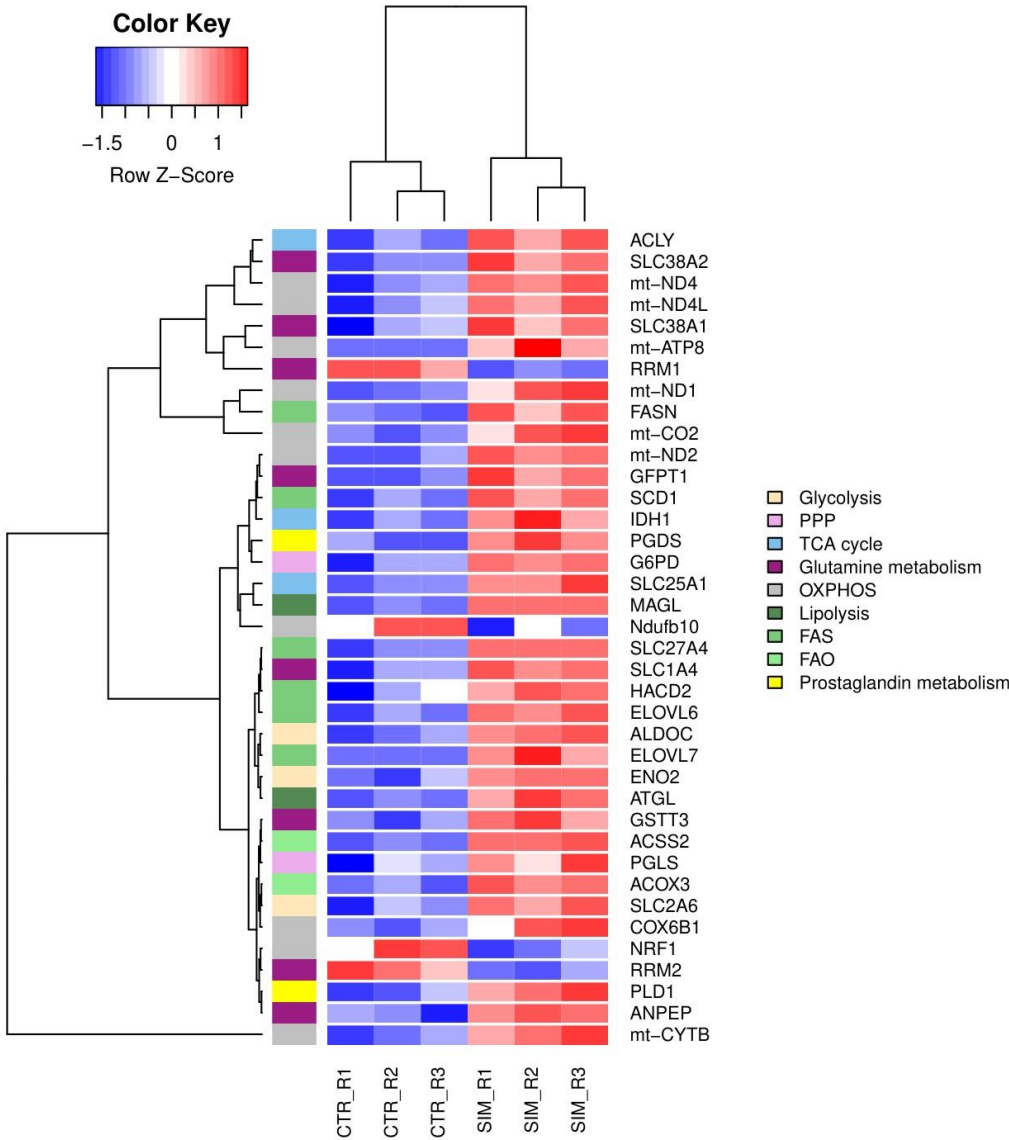


Figure 7. Supervised hierarchical clustering analysis of DEG in the control *versus* SIM-treated group in B16.F10 melanoma cells under normoxic conditions. Heatmap Clustering of 31 genes was generated using normalized FPKM values. Each column represents a biological replicate/group (CTR_R1, CTR_R2, CTR_R3 replicates of the gene expression in untreated cells and SIM_R1, SIM_R2, SIM_R3 replicates of the gene expression in SIM-treated cells), and each row represents a specific transcript. The level of each gene's expression in a single sample is displayed in accordance with the color scale, red color represents relative increase in transcript abundance (positive Z-scores; $0 < Z\text{-score} \leq 1.5$), blue color represents relative decrease (negative Z-scores; $-1.50 \leq Z\text{-score} < 0$), while white

indicates median gene expression (Z-score close to zero). Names of the corresponding metabolic pathway for each gene were listed on the right of the heatmap. Genes with $FR \pm 1.5$, $p < 0.05$, FDR 20% were considered differentially expressed. Abbreviations: ACLY: ATP citrate lyase; ACS2: acyl-CoA synthetase short-chain family member 2; ACOX3: acyl-CoA oxidase 3; ANPEP: alanyl aminopeptidase; ATGL: adipose triglyceride lipase; COX6B1: cytochrome c oxidase subunit 6B1; ELOVL6: elongation of very long chain fatty acids protein 6; ELOVL7: elongation of very long chain fatty acids protein 7; ENO2: enolase 2; FASN: fatty acid synthase; GFPT1: glutamine-fructose-6-phosphate transaminase 1; GSTT3: glutathione S-transferase theta 3; HACD2: 3-hydroxyacyl-CoA dehydratase 2; IDH1: isocitrate dehydrogenase 1; MAGL: monoacylglycerol lipase; mt-ATP8: mitochondrial ATP synthase subunit 8; mt-CO2: mitochondrial cytochrome c oxidase subunit 2; mt-CYTB: mitochondrial cytochrome b; mt-ND1: mitochondrial NADH dehydrogenase subunit 1; mt-ND2: mitochondrial NADH dehydrogenase subunit 2; mt-ND4: mitochondrial NADH dehydrogenase subunit 4; mt-ND4L: mitochondrial NADH dehydrogenase subunit 4L; NRF1: nuclear respiratory factor 1; PGLS: 6-phosphogluconolactonase; PGDS: prostaglandin D2 synthase; PLD1: phospholipase D1; RRM1: ribonucleotide reductase subunit M1; RRM2: ribonucleotide reductase subunit M2; SCD1: stearoyl-CoA desaturase 1; SLC2A6: solute carrier family 2 member 6; SLC25A1: solute carrier family 25 member 1; SLC27A4: solute carrier family 27 member 4; SLC38A1: solute carrier family 38 member 1; SLC38A2: solute carrier family 38 member 2; SLC1A4: solute carrier family 1 member 4; SLC38A1: solute carrier family 38 member 1.

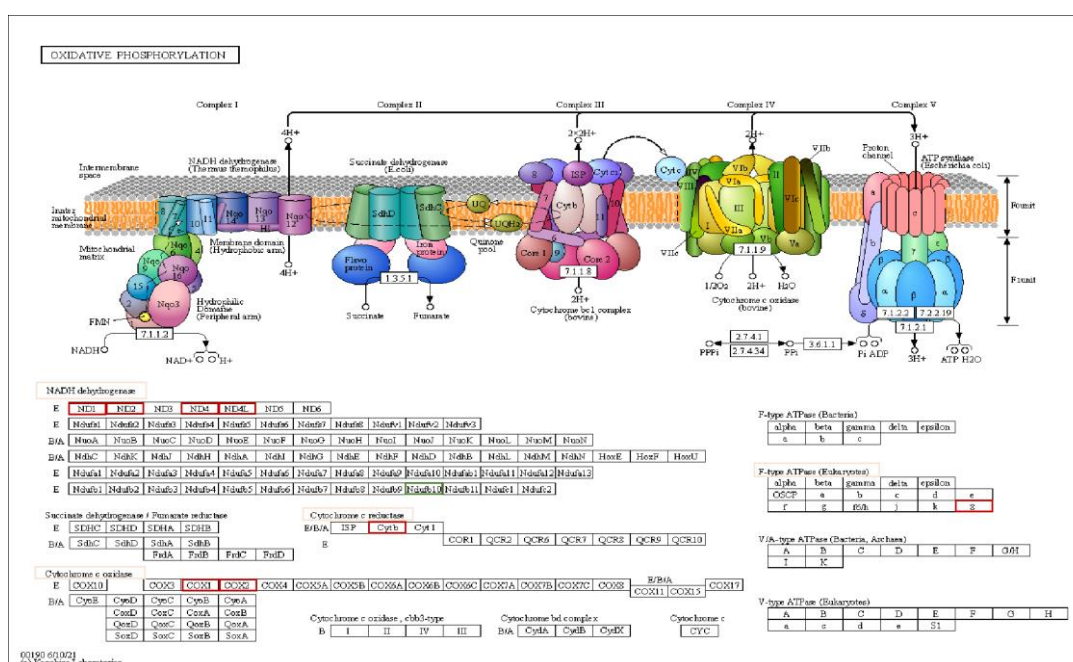


Figure 8. Graphical visualization *via* KEGG pathways of significant DEG in SIM-treated cells compared to control cells involved in OXPHOS. Highlighted by red squares are genes that were up-regulated, while green indicate down-regulated genes in every complex of the respiratory chain system. Pink squares show the gene complexes whose expression had been influenced by SIM. Genes with $FR \pm 1.5$, $p < 0.05$ and FDR 20% were considered DEG. Abbreviations: COX6B1: cytochrome c oxidase subunit 6B1; DEG: differentially expressed genes; FDR: false discovery rate; FR: fold regulation; KEGG: Kyoto Encyclopedia of Genes and Genomes; mt-ATP8: mitochondrial ATP synthase subunit 8; mt-CO2: mitochondrial cytochrome c oxidase subunit 2; mt-CYTB: mitochondrial cytochrome b; mt-ND1: mitochondrial NADH dehydrogenase subunit 1; mt-ND2: mitochondrial NADH dehydrogenase subunit 2; mt-ND4: mitochondrial NADH dehydrogenase subunit 4; mt-ND4L: mitochondrial NADH dehydrogenase subunit 4L; Ndufb10: NADH dehydrogenase:Ubiquinone Oxidoreductase Subunit B10; OXPHOS: oxidative phosphorylation.

4. Discussion

The present work provides a follow-up of our previous findings that inhibition of the constitutive expression of HIF-1 in B16.F10 murine melanoma cells is one of the main actions behind the cytotoxicity caused by SIM on these cancer cells (Alupe et al, 2014; Licarete et al, 2017). As HIF-1 is an essential regulator of cancer cell metabolism under hypoxia, this study aimed to investigate whether SIM-induced reduction of the normoxic levels of HIF-1 might also affect melanoma cell metabolism (DeBerardinis and Chandel, 2016; Munir et al, 2019; Shiratori et al, 2019). Since the translation of subunit α of HIF-1 (HIF-1 α) is tightly linked to Akt/mTOR signaling (Harada et al, 2009) and Akt is also a well-known central regulator of cancer metabolic reprogramming (Semenza et al, 1996; Kuphal et al, 2010; Kwong and Davies, 2013; Koch et al, 2015; Choi et al, 2016; Li et al, 2008; Xiao et al, 2017; Tian et al, 2023; Harada et al, 2009; Majumder et al, 2004), we also explored SIM effects on Akt mRNA and protein production in melanoma cells. Our data proved that pAkt/total Akt is also reduced after SIM-administration compared to control (Figure 3), these findings being consistent with previous reports (Ghosh-Choudhury et al, 2010; Hwang et al, 2011; Park et al, 2013). As SIM inhibited these two connected regulatory proteins, we further analyzed whether the energy and biosynthesis metabolic pathways of melanoma cells (Figures 2-10) were affected. In tight relation with the inhibitory effect of SIM on rate-limiting enzyme of cholesterol biosynthesis pathway (Endo, 2008; Taylor et al, 2013; Beckwitt et al, 2018), the expression of the genes associated with isoprenoids production was assessed. Our transcriptomic results might suggest that inhibitory effects exerted by SIM on melanoma cell cholesterol metabolism were counteracted via upregulation of mRNA expression of genes encoding enzymes for isoprenoids production which finally will enhance cancer cell survival rates (Bjarnadottir et al, 2015; Kimbung et al, 2016; Lazaro-Mixteco et al, 2022). With regard to glucose metabolism, although no changes at mRNA levels for PFK1 and LDH (Supplementary Table 1) were noted in SIM-treated cells, catalytic activities of PFK1 and LDH were decreased while protein level of GLUT1 was elevated (Figure 9 A,B; Figure 10 A,B), probably signaled through HIF-1-independent mechanisms (Vander Heiden et al, 2001; Elstrom et al, 2004; Düvel et al, 2010; Jacobs et al, 2008; Yun et al 2009). Moreover, despite increased G6PD and PGD mRNA expression (Table 4; Figures 6, 7) after SIM administration, catalytic activity of G6PD was suppressed (Figure 9C), indicating a decrease in the glucose use via PPP. These data might suggest that in SIM-treated cells, low rates of glycolysis and PPP might be compensated via an enhanced influx of glucose via GLUT1 and reduced lactate production (Le et al, 2010; Haugrud et al, 2014; Circu et al, 2017; Li and Cui, 2023) which in turn, will provide enough pyruvate to mitochondria ensuring metabolites for TCA cycle activity. Restoring activity of the TCA cycle was also supported by the data regarding the substantial increase in the catalytic activity of CS (Figure 9D) as well as mRNA levels for IDH1 and glutamine metabolism (Figures 2, 6, 7, Table 4, and Supplementary Table 1) (Chen et al, 2019; Ding et al, 2021; Aurora et al, 2022; Song et al, 2017). Nevertheless, the reduction of mt-ND1 protein levels (Figure 10A,C) as well as overexpression of mRNA for SLC25A1 suggested that biosynthetic role of TCA was prioritized over its energy role in SIM-treated cells compared to untreated cells (Table 4; Figures 6, 7) (Simonnet et al, 2002; DeBerardinis et al, 2008; Lin et al, 2012; Chen et al, 2014; Mycielska et al, 2006; Cai et al, 2020; Isidoro et al, 2004; Chae et al, 2016). In addition to the efflux of citrate via SLC25A1 from mitochondrion to cytosol, SIM treatment favored the transcription of lipolysis enzymes (such as, MAGL and ATGL) (Table 4 and Figures 6, 7) which linked to cancer cell aggressiveness (Hu et al, 2014; Nomura et al, 2010; Zhu et al, 2016), via accumulation of available FAs to support the synthesis of lipid-derived signaling molecules (Wymann and Schneider, 2008). Previous studies proved that in hypoxia, Akt promotes FAs and lipid synthesis by favoring FASN and ACLY availability (by transcription activation and protein phosphorylation) (Berwick et al, 2002; Bauer et al, 2005). In normoxia, FASN expression was favored both at mRNA and protein level in SIM treated group compared to the control (Table 4, Figure 10 A,D), suggesting high rates of FAS which give anti-apoptotic advantages contributing to chemoresistance (Yang et al, 2011; Papaevangelou et al, 2018). However, we did not identified a negative-feedback of FASN over pAkt, or *vice versa* in normoxic conditions, compared to the published data from hypoxic conditions (Wang et al, 2013) (Figure 3 A,C; Figure 10 A,D). Also, the elevated ACLY mRNA expression in SIM-treated cells supported our hypothesis about the role of

TCA cycle to produce citrate for *de novo* FAS (Table 4, Figure 6,7). For protective effects against lipid peroxidation and ER-related stress, SIM-treated cells boosted both SCD1 mRNA and protein level to reduce SFAs level and increase UFAs level (Table 4; Figures 6,7 and 10 A,E) (Gonzalez et al, 2018; Piccolis et al, 2019; Ackerman et al, 2018) and upregulated ELOVL genes to favor FAE (Table 4, Figures 6 and 7) (Su et al, 2018; Naganuma et al, 2011). No significant increase in mitochondrial or peroxisomal FAO was seen in the SIM-treated group (Figure 10 A,F), despite the reduced HIF-1 and pAkt protein levels, which prevent lipid catabolism in hypoxia (Szutowicz et al, 1974; Flavin et al, 2010; Fazolini et al, 2015; Zhang, 2015; Huang et al, 2018). This suggested cancer cells might need less energy to survive than previously assumed (Bartman et al, 2023). Our findings point to upregulation of prostaglandin synthesis post-treatment, considering the favored lipid metabolism that leads to high PUFAs availability (Koundouros and Poulogiannis, 2020) and the increased PGD2 and PLD1 mRNA levels (Table 4; Figure 7). The dual pro/anti-inflammatory role of PGDS remains debatable (Breyer et al, 2001; Rajakariar et al, 2007; Fukuoka et al, 2015; Dash et al, 2022; Jiang et al, 2020), yet PGDS could serve as a significant diagnostic marker and treatment target (Jiang et al, 2020).

Altogether, our study indicated that under normoxia, SIM inhibition of HIF-1 and Akt levels induced different effects on B16.F10 murine melanoma cell metabolism than those described previously under hypoxia (Phan et al, 2014). Our data suggested that SIM treatment orientated lipid metabolism to support isoprenoid and prostaglandin synthesis which might favor melanoma cell survival and aggressiveness. To counteract SIM induced metabolic plasticity, combination therapies should be explored (Yakes et al, 2011; Telang et al, 2014; Jones et al, 2019), potentially using 15-prostaglandin dehydrogenase (15-PGDH) to favor prostaglandin turnover (Backlund et al, 2008) and L-glutamine analogue L- γ -glutamyl-p-nitroanilide (GPNA) to inhibit glutamine transporters (Hassanein et al, 2013; Choi and Park, 2018).

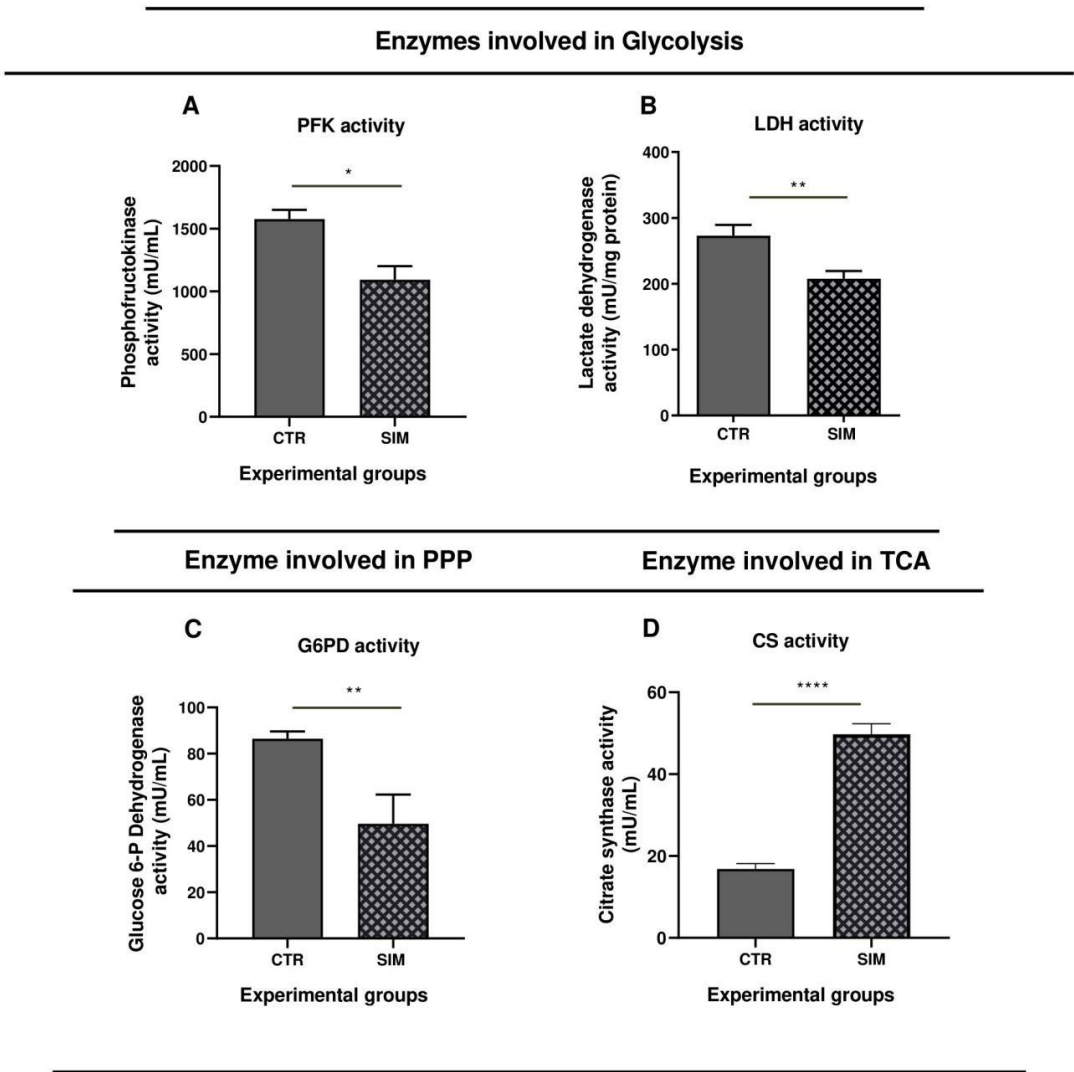


Figure 9. Effects of SIM on the catalytic activities of specific enzymes in B16.F10 melanoma cells. Graphical representations of catalytic activities of different enzymes in lysates of B16.F10 cells treated with 3.5 μ M SIM for 24h in normoxia compared to control: **(A)** PFK activity expressed as mU/ml; **(B)** LDH activity expressed as mU/mg protein; **(C)** G6PD activity expressed as mU/ml; **(D)** CS activity represented as mU/ml. All data were expressed mean $\pm$ SD of at least three independent experiments. T-test was used for statistical analysis of the data; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. **Abbreviations:** CS: citrate synthase; CTR: control group; G6PD: glucose-6-phosphate dehydrogenase; LDH: lactate dehydrogenase; PFK: phosphofructokinase; SIM: simvastatin treated group. .

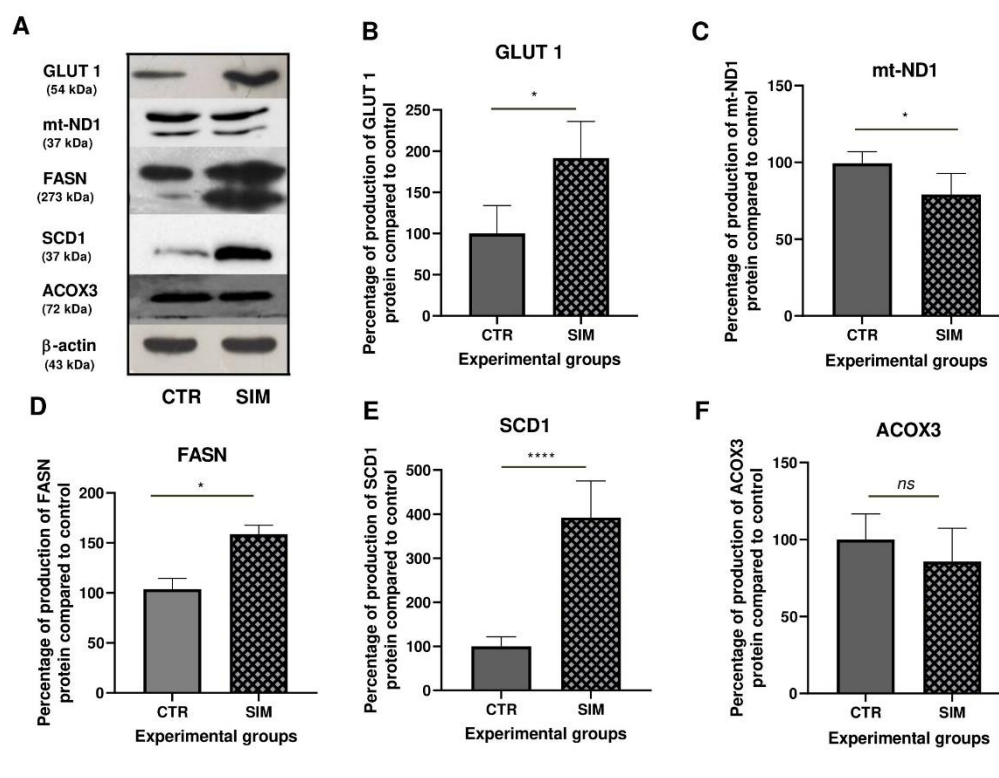


Figure 10. Western blot analysis of the effects of SIM on specific protein levels in B16.F10 melanoma cells. Western blot images (A) and their corresponding graphs showing the percentage of protein levels after administration of 3.5 μ M SIM for 24h, in normoxia, as compared to control levels of the same proteins; (B) GLUT1 protein expression; (C) mt-ND1 protein expression; (D) FASN protein expression; (E) SCD1 protein expression; (F) ACOX3 and β -actin was used as loading control. All data were expressed mean $\pm$ SD of at least three independent experiments. T-test was used for statistical analysis of the data; *, $p < 0.05$; **, $p < 0.01$; ns: not significant. **Abbreviations:** ACOX3: acyl-CoA oxidase 3; β -actin: beta-actin; CTR: control group; FASN: fatty acid synthase; GLUT1: glucose transporter 1; mt-ND1: mitochondrially encoded NADH dehydrogenase 1; SCD1: stearoyl-CoA desaturase 1; SIM: simvastatin treated group.

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