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Posted Date: 14 November 2024

doi: 10.20944/preprints202411.1006.v1

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Article

TGFβ1 Restores Energy Homeostasis of Human Trophoblast Cells Under Hyperglycemia *In Vitro* by Inducing PPARγ Expression, AMPK Activation, and HIF1α Degradation

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Abstract: Elevated glucose levels at the fetal-maternal interface are associated with placental trophoblast dysfunction and increased incidence of pregnancy complications. Trophoblast cells predominantly utilize glucose as an energy source, metabolizing it through glycolysis in the cytoplasm and oxidative respiration in the mitochondria to produce ATP. The TGFβ1/SMAD2 signaling pathway and the transcription factors PPARγ, HIF1α, and AMPK are key regulators of cell metabolism and known to play critical roles in extravillous trophoblast cell differentiation and function. While HIF1α promotes glycolysis over mitochondrial respiration, PPARγ and AMPK encourage the opposite. However, the interplay between TGFβ1 and these energy-sensing regulators in trophoblast cell glucose metabolism remains unclear. This study aimed to investigate whether and how TGFβ1 regulate energy metabolism in trophoblast cells exposed to normal and high glucose conditions. The trophoblast JEG-3 cells were incubated in normal (5 mM) and high (25 mM) glucose conditions for 24 h in the absence and the presence of TGFβ1. The protein expression levels of phosphor (p)-SMAD2, GLUT1/3, HIF1α, PPARγ, p-AMPK, and specific OXPHOS protein subunits were determined by western blotting, and ATP and lactate production by bioluminescent assay kits. JEG-3 cells exposure to 25 mM glucose decreased ATP production but did not affect lactate production. These changes led to a reduction in the expression levels of GLUT1/3, mitochondrial respiratory chain proteins, and PPARγ, coinciding with an increase in HIF1α expression. Conversely, TGFβ1 treatment at 25 mM glucose reduced HIF1α expression while enhancing the expression levels of GLUT1/3, PPARγ, p-AMPK, and mitochondrial respiratory chain proteins, thereby rejuvenating ATP production. Our findings reveal that high glucose conditions disrupt cellular glucose metabolism in trophoblast cells by perturbing mitochondrial oxidative respiration and decreasing ATP production. Treatment with TGFβ1 appears to counteract this trend probably by enhancing both glycolytic and mitochondrial metabolism, suggesting a potential regulatory role of TGFβ1 in placental trophoblast cell glucose metabolism.

Keywords: adenosine triphosphate; diabetes; extra-villous trophoblast; glucose metabolism; glycolysis; hyper-glucose; oxidative phosphorylation; placenta; transforming growth factor beta 1

1. Introduction

Elevated levels of glucose have a significant impact on maternal and fetal interactions as well as on the production of essential hormones that support pregnancy [1]. Studies have shown that high glucose levels are linked to a decrease in the number of active mitochondria and reduced ATP production, which can negatively affect trophoblast function [2, 3]. This may result in an increased incidence of pregnancy complications such as preeclampsia, congenital malformations, and miscarriage, posing significant health risks to both pregnant women and fetuses [4].

The placenta is a highly metabolically active organ fulfilling the bioenergetic and biosynthetic needs to support its own rapid growth and that of the fetus [5]. Placental structures consist of specialized epithelial cell types known as trophoblast cells, which are located at the maternal-fetal interface [6, 7]. Human trophoblasts arise from the trophoctoderm, which, after implantation, differentiates into cytotrophoblast (CT), villous syncytiotrophoblast (VST), and extravillous trophoblast (EVT) cells [6, 7]. VST cells form a multinucleated layer on the placental surface and play key roles in hormone secretion, nutrient transport, gas exchange, immunotolerance, and pathogen resistance [8, 9]. EVT cells migrate, invade, and embed placental villi into the maternal decidua to anchor the placenta [10, 11].

Trophoblasts use glucose as the primary energy substrate. In the absence of appreciable gluconeogenesis, placental glucose transport is the only supply for the fetus [12]. In trophoblasts, glucose is metabolized through a multistep process. The first step, glycolysis, occurs in the cytoplasm and involves the conversion of one glucose molecule to two pyruvate molecules, resulting in a net gain of two ATP molecules [1]. Under anaerobic conditions, pyruvate can be reversibly converted to lactate by the enzyme lactate dehydrogenase (LDH). Pyruvate is typically transported into the mitochondria for further degradation in the TCA cycle, where it is oxidized to acetyl-CoA [5]. This process occurs iteratively, generating one ATP molecule for each acetyl-CoA molecule produced, but also generating reduced nicotinamide adenine dinucleotide ($\text{NADH}+\text{H}^+$) and flavin adenine dinucleotide (FADH_2). These reduced cofactors act as electron carriers to generate a proton gradient that drives ATP production through oxidative phosphorylation (OXPHOS) in the electron transport chain [5].

Cellular glucose metabolism is regulated by complex signaling pathways that involve various biological factors. Transforming growth factor beta 1 ($\text{TGF}\beta 1$), a multifunctional cytokine, is one of these factors [13]. It operates through the SMAD2/SMAD3 pathway to regulate the expression of genes involved in different cellular functions. According to previous research, $\text{TGF}\beta 1$ regulates the activity of essential enzymes and transporters, such as GLUT1 and the hexokinase HK2, which play crucial roles in glucose uptake and glycolysis in different cell types [13]. However, $\text{TGF}\beta 1$ is also known to induce metabolic reprogramming by promoting mitochondrial ATP production in stromal cells [14]. $\text{TGF}\beta 1$ expression is detected at the human maternal-fetal interface during early pregnancy, playing crucial roles in regulating immune cell function and maintaining immune homeostasis [15]. Moreover, $\text{TGF}\beta 1$ plays significant roles in the regulation of trophoblast function, particularly trophoblast invasion and differentiation [15].

Transcription factors, such as hypoxia-inducible factor (HIF), AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor gamma ($\text{PPAR}\gamma$), are considered energy sensors that play significant roles in regulating cellular metabolism [16-18]. HIFs are transcriptional heterodimer complexes, consisting of an inducible α subunit ($\text{HIF1}\alpha$, $\text{HIF2}\alpha$ and $\text{HIF3}\alpha$) and constitutively expressed β subunits [16]. Under hypoxic conditions, the α/β heterodimer binds to the hypoxia response elements (HREs) of the target genes. Under normal oxygen levels, proline residues in $\text{HIF1}\alpha$ and $\text{HIF2}\alpha$ are hydroxylated by prolyl hydroxylase domain (PHD) proteins, allowing their recognition by a ubiquitin ligase leading to proteasomal degradation [17]. Studies have demonstrated that $\text{HIF1}\alpha$ is significantly expressed in the low-oxygen environment of the placenta during early gestation where it plays a crucial role in placental development and function [18]. However, sustained $\text{HIF1}\alpha$ expression after 9 weeks of gestation can lead to trophoblast cells failing to differentiate from a proliferative to an invasive phenotype, shallow invasion of trophoblasts, and insufficient myometrial spiral artery transformation, which are strongly associated with pregnancy

complications [19]. On the other hand, AMPK functions as a primary regulator of energy metabolism. AMPK is activated in response to energy stress, which is characterized by increased levels of cellular AMP, ADP, or Ca^{2+} and a decline in ATP production [20]. Research has shown that AMPK plays a crucial role in maintaining the balance between trophoblast invasion and survival by controlling glucose metabolism [21]. Furthermore, elevated blood glucose levels trigger AMPK activation in the placentas of pregnant women with gestational diabetes, indicating that AMPK plays a key role in regulating glucose metabolism to maintain energy balance [22]. Finally, PPAR γ is a crucial nuclear hormone receptor whose action is mediated through the heterodimerization of PPAR γ with the retinoid-X-receptor (RXR) upon activation. According to previous studies, PPAR γ is a crucial component for proper progression of pregnancy, as it encompasses placental formation, fetal development, and labor [23]. Additionally, PPAR γ activators, which include fatty acids and lipid metabolites, are elevated in normal pregnancies, suggesting that PPAR γ may play a part in regulating maternal metabolism and immune functions during pregnancy [24].

Studies suggest that HIF1 α promotes glycolysis through regulation of the glycolytic enzymes hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2), and the glucose transporters GLUT1 and GLUT3, facilitating cellular glucose uptake [25]. Furthermore, glycolysis and glucose uptake affect the stability and activation of HIF1 α in human pharyngeal carcinoma and fibrosarcoma cells and rat cardiac myocytes [25]. Conversely, AMPK activation leads to the activation of catabolic pathways that generate ATP while simultaneously inhibiting anabolic, biosynthetic pathways that consume ATP [20]. When PPAR γ is activated, it initiates a series of events that ultimately lead to the regulation of glucose and lipid metabolisms. Specifically, PPAR γ regulates genes associated with glucose transporters, mitochondrial biogenesis, fat storage, and fat transport [26].

The interactions between high-glucose levels and TGF β 1 have been investigated in various cell lines, and the results have demonstrated that TGF β 1 signaling affects glucose metabolism by increasing glucose uptake, controlling glycolytic enzymes, altering lactate production, and influencing oxidative phosphorylation [13, 27]. However, there is no evidence regarding the effect of TGF β 1 on energy metabolism in trophoblast cells. Precisely, the interplay between TGF β 1 and the energy-sensing regulators: HIF1 α , PPAR γ , and AMPK.

The aim of the present study was to investigate the potential regulatory function of TGF β 1 in counteracting metabolic disruptions caused by high-glucose conditions in trophoblast cells. By examining the expression or activation levels of SMAD2, HIF1 α , PPAR γ , AMPK, GLUT1, GLUT3, and specific mitochondrial respiratory chain protein subunits, as well as assessing the levels of ATP and lactate production, we aimed to explore the extent to which TGF β 1 may play a role in regulating the effects of high-glucose conditions on trophoblast cells.

2. Materials and Methods

2.1 Materials

Cell culture media and reagents such as fetal bovine serum (FBS; #090-110) and Hank's Balanced Salt Solution (HBSS, #311-512-CL) were obtained from Wisent (St-Bruno, QC, Canada). Cell culture plates and flasks were purchased from Corning Inc. (Corning, NY, USA). TGF β 1 cytokine was purchased from Peprotech (Montreal, QC, Canada). Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and monoclonal peroxidase-conjugated mouse anti- β -actin antibody were from Sigma Chemical Company (Oakville, ON, Canada). Protease and phosphatase inhibitors cocktail EDTA-free and the PierceTM NE-PERTM nuclear and cytoplasmic extraction reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). VH 298, a cell-permeant inhibitor of von Hippel-Lindau disease tumor suppressor (VHL; #700410) was from Cayman Chemicals, Ann Arbor, MI, USA. Rabbit polyclonal antibodies (Abs) targeting PPAR γ (#2435), HIF1 α (#36169), p-SMAD2 (pS465/467; #3108), SMAD2 (#5359), p-AMPK α (pThr172; #2531), AMPK (#2532), and Lamin B1 (#13435), GLUT1 (#12939) were purchased from Cell Signaling Technology (Danvers, MA, USA). The Total OXPHOS Rodent WB Antibody Cocktail (ab110413) was sourced from Abcam (Cambridge, UK), and the mouse monoclonal GLUT3 Abs (Sc-74399) from Santa Cruz biotechnology (Cambridge,

MA, USA). Rabbit polyclonal β -tubulin Abs were purchased from Abcam (#Ab6046; Waltham, MA, USA). All Abs were used at a 1:1000 dilution except for GLUT3 (1:500) in phosphate-buffered saline (PBS) solution containing 5 % BSA (PBS/5 % BSA). The horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG Abs (1:5000 dilution) were obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). The chemiluminescence detection kit was purchased from FroggBio (#CCH365; Concord, ON, Canada).

2.2. Cell culture

The human placental choriocarcinoma JEG-3 cell line (ATCC #HTB-36; Rockville, MD, USA) was grown in RPMI-1640 cell culture media supplemented with 10 % heat-inactivated FBS, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μ g/mL gentamicin. Cell culture workflow, cell passaging, and cell subculturing were performed as previously described [8-10]. For further experimental needs, cells were detached using trypsin, counted, and subcultured in 24- or 96-well cell culture plates. All experiments were restricted to using JEG-3 cells from passages 8 to 15 to avoid any changes in cell behavior during the study.

2.3. ATP detection

The metabolic activity of JEG-3 cells was evaluated via quantitative measurement of total cellular ATP levels and achieved using an ATP detection assay kit (#700410; from Cayman Chemicals, Ann Arbor, MI, USA). Briefly, at the indicated time points, cells were lysed in ATP sample buffer, and properly diluted to ensure the luminescence was within the linear range of the ATP standard curve. Cell lysates were incubated with a mixture containing D-Luciferin and Luciferase at room temperature for 20 min. The luminescence intensity was recorded at 570 nm using a microplate reader (BioTek Synergy HT; BioTek® Instruments, Inc., Winooski, VT, USA). According to the manufacturer protocol, total ATP concentration, expressed in nM, was determined using the ATP detection standard.

2.4. Lactate Detection

The glycolytic activity of JEG-3 cells was determined using the Lactate-Glo Assay (#J5021, from Promega, Madison, WI, USA). In this bioluminescent assay, lactate dehydrogenase (LDH) utilizes extracellular lactate, derived from glycolysis, and NAD^+ to produce pyruvate and NADH. In the presence of NADH, a pro-luciferin reductase substrate is converted by the reductase to luciferin, which is then used in a luciferase reaction to produce light. The light emitted in this reaction is directly proportional to the concentration of lactate in the culture medium sample. Briefly, at the indicated time points, 10 μ L of cell-free culture medium samples were firstly diluted with 490 μ L HBSS. Then, 50 μ L diluted medium sample and 25 μ L lactate detection reagent was mixed in 96-well plates and incubated for 60 min at room temperature. The luminescence intensity was recorded at 570 nm using the BioTek Synergy HT microplate reader. According to the technical manual, relative light unit and lactate titration curve were used to determine lactate concentration, expressed in nM.

2.5. Subcellular Fractionation

JEG-3 cells at a cell density of 450×10^3 cells/2 mL/well were seeded into 6-well plates in 10 % FBS-RPMI-1640 cell culture media overnight. Cells were starved without FBS overnight, and then cultured for 24 h. After incubation period, cytoplasmic and nuclear protein extracts were prepared according to the instructions of the Pierce™ NE-PER® nuclear and cytoplasmic extraction kit (#78835) and analyzed by western blot.

2.6. Proteins Immunodetection

JEG-3 cells were seeded for 24 h at two different cell densities, 450×10^3 cells/2 mL/well into 6-well plates and 150×10^3 cells/500 μ L/well into 24-well plates. Cells were starved in cell culture media without FBS overnight. Cells were then used in different experimental conditions. At the indicated

time points, cells protein samples were resolved using SDS-PAGE under reducing conditions, and then transferred into PVDF membranes as described [8-10]. Blots were probed overnight at 4 °C with rabbit polyclonal primary Abs (1:1000 dilution) against total (t) or phosphorylated (p) forms of SMAD2, HIF1 α , PPAR γ , GLUT1, and AMPK proteins; and a mix of mouse polyclonal primary Abs against mitochondrial respiratory chain protein subunits, and mouse monoclonal Abs against GLUT3. Membranes were then incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG Abs at 1:5000 dilution for 1 hour at room temperature. β -actin (1:40000 dilution), and β -tubulin and Lamin B1 (1:1000 dilutions) were used as loading controls. The detected proteins were visualized as described [8-10].

2.7. Statistical Analysis

Data collection and statistical analysis were performed using Prism software (San Diego, CA, USA), as previously described [8-10]. Briefly, data from at least three independent experiments were expressed as mean \pm SD. A one-way ANOVA followed by Bonferroni post-tests were performed to assess the statistical correlation of data between groups. Tukey tests were performed to analyze the null hypothesis of no difference of means between two groups. A threshold of significance at $p \leq 0.05$ was considered to indicate statistical significance.

3. Results

3.1 The interplay between glucose concentration and metabolic changes in human trophoblast cells

To determine the effect of glucose concentration on metabolic regulation in the human trophoblast cell line JEG-3, cells were incubated for 24 h with increasing concentrations of glucose: 5 mM (control), 10 mM, 15 mM, and 25 mM. We assessed both ATP and lactate production, as these markers are useful for evaluating energy and carbohydrate metabolism, respectively. JEG-3 cells growing in increasing glucose concentrations exhibited a significant decrease in total ATP production immediately after the glucose concentration was doubled from 5 mM to 10 mM (Figure 1A). This decline continued gradually, reaching the lowest ATP concentration after a 24-h incubation period with 25 mM glucose. Regarding lactate production (Figure 1B), no significant changes in lactate production was observed, regardless of the glucose concentration. These findings suggest that increasing glucose concentrations do not affect the glycolytic pathway but probably induces alterations in ATP production within the mitochondria of JEG-3 cells.

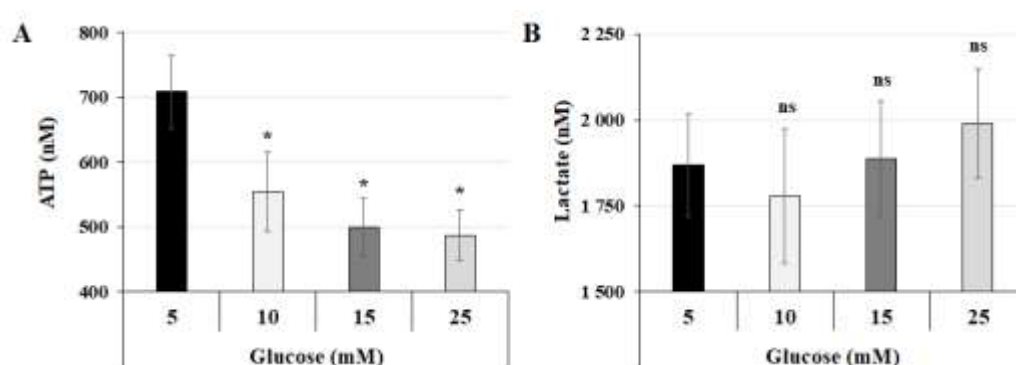


Figure 1. High-glucose concentration decreases total ATP production without affecting lactate production: (A,B) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (10, 15, and 25 mM). (A) Quantitation of ATP production (n = 3) was assessed using ATP luminescence detection assay kit. (B) Quantitation of lactate production (n = 3) was assessed using Lactate-Glo™ assay kit. Data are expressed as nM of ATP and lactate. * $p < 0.05$ indicates a significant difference compared to 5 mM-glucose group, and ns = nonsignificant difference.

3.2. Effect of High-Glucose Concentration on Mitochondrial Respiratory Chain Proteins Expression

Because high-glucose condition is known to reduce ATP synthesis and mitochondrial function in trophoblast cells [2, 3], and that variation in the expression of mitochondrial respiratory chain proteins is linked to enhanced [28] or lowered [29] cellular ATP synthesis levels, we evaluated the expression of mitochondrial respiratory chain proteins via western blot analysis (Figure 2A). JEG-3 cells were incubated for 24 h with glucose at either 5 mM or 25 mM — the concentration that mimic conditions of hyperglycemia *in vivo*. The results showed that incubating JEG-3 cells with 25 mM glucose significantly reduced the expression of all mitochondrial respiratory chain proteins subunits: CI-NDUF88 (2-fold), CII-SDHB (1.9-fold), CIII-UQCRC2 (1.6-fold), and CIV-MTCO1 (1.3-fold) (Figure 2B). This indicates that cell growing in high-glucose conditions leads to alterations in mitochondrial function.

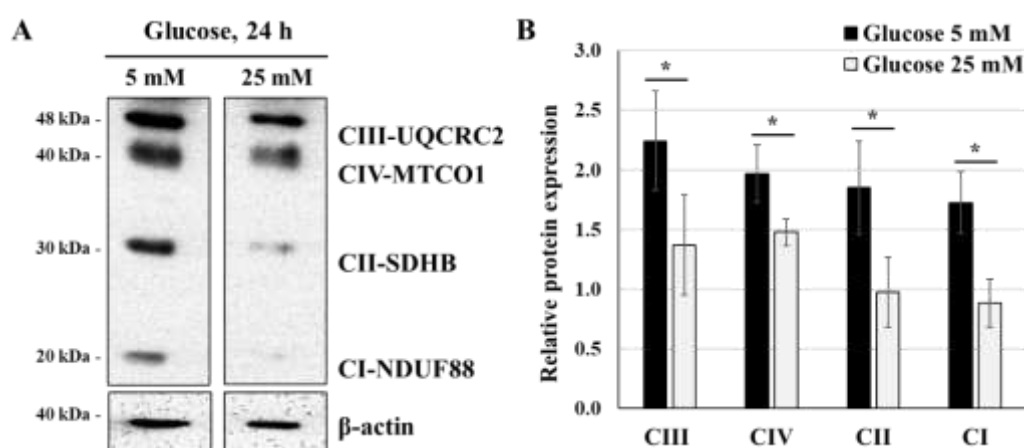


Figure 2. High-glucose concentration decreases the expression of mitochondrial respiratory chain proteins: (A,B) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM). (A) Representative images of OXPHOS complexes protein subunits detection and β -actin, as assessed by western blot. (B) Graphical analysis showing the expression of different mitochondrial complexes proteins subunits relative to β -actin at 5 mM and 25 mM glucose (n = 3). * $p < 0.05$ denotes a significant difference between the cell groups.

3.3. Effect of High-Glucose Concentrations on the Expression of HIF1 α and PPAR γ

To better understand the mechanism by which high-glucose concentration alters ATP production and mitochondrial metabolism, we aim to investigate the expression of HIF1 α and PPAR γ , two key transcription factors involved in the regulation of carbohydrate metabolism. HIF1 α , a transcription factor induced by hypoxia, is also known to regulate glycolytic metabolism [30]. PPAR γ is a key regulator of energy metabolism, particularly in mitochondrial biogenesis [31]. In this set of experiments, proteins from cytoplasmic and nuclear compartments were extracted from JEG-3 cells which were incubated with either normal-glucose (5 mM) or high-glucose (25 mM) concentrations for 24 h. The results (Figure 3A,B) showed that growing conditions in high-glucose concentration induced a decrease in the nuclear expression of PPAR γ (1.7-fold decrease) compared with the normal-glucose condition. Conversely, nuclear expression of HIF1 α appears to be increased (1.7-fold increase) in JEG-3 cells after a 24-h incubation period with high-glucose concentration compared with 5 mM glucose (Figure 3A,B). Taken together, these results indicate that high-glucose concentration induces alterations in the regulation of energy metabolism in the human trophoblast JEG-3 cells. These alterations appear to be mediated through differential expression of the energy metabolic sensors HIF1 α and PPAR γ .

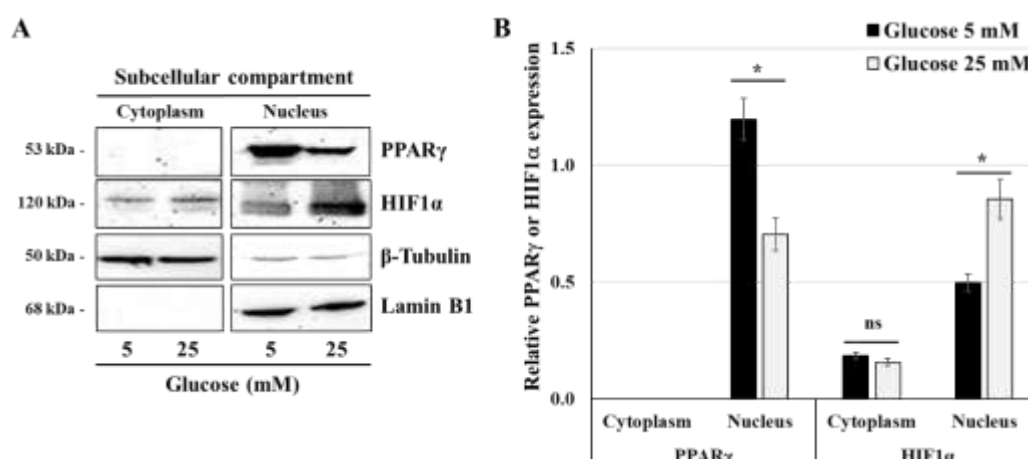


Figure 3. High-glucose concentration differentially regulates the nuclear expression of PPAR γ and HIF1 α : (A,B) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentration (25 mM). (A) Representative images of PPAR γ , HIF1 α , β -tubulin, and Lamin B1 in the cytoplasmic and nuclear compartments of JEG-3 cells, as assessed by western blot. (B) Graphical analysis showing the relative expression of HIF1 α and PPAR γ in the cytoplasm and the nucleus at 5 mM and 25 mM glucose (n = 3). * $p < 0.05$ denotes a significant difference between the cell groups, and ns = nonsignificant difference.

3.4. Effect of Glucose Concentrations on The Activation of TGF β 1 Signaling Pathway

In human trophoblast cells, TGF β 1 is recognized as a key pregnancy-related cytokine and regulates various aspects of trophoblast function such as differentiation and proliferation [32]. TGF β 1 is also known to be a main regulator of metabolism in immune cells, such as macrophages, by promoting mitochondrial metabolism over glycolytic metabolism [33]. In this set of experiments, we aimed to assess the effect of TGF β 1 on the metabolic dysregulation induced by growing conditions in high-glucose concentration. Initially, we evaluated the impact of different glucose concentrations on the phosphorylation of SMAD2, a key signal transduction molecule induced by TGF β 1. Our preliminary data indicates that SMAD2 activation is induced as early as 5 min after TGF β 1 stimulation, with a peak at 60 min and a continuing decline up to 120 min (data non shown). JEG-3 cells were initially incubated for 24 h with increasing concentrations of glucose (5, 10, 15, and 25 mM). Then, cells were stimulated for 30 min with culture media alone (control) or with 50 ng/ml TGF β 1. The results, showed in Figures 4A,B indicate that stimulation with TGF β 1 during 30 min results in significant induction of SMAD2 phosphorylation, with greater induction observed when the cells were incubated with 20 mM (4.3-fold increase) and 25 mM (4.6-fold increase) glucose. Overall, these results suggest that TGF β 1 signaling can be influenced in trophoblast JEG-3 cells when incubated in high-glucose conditions.

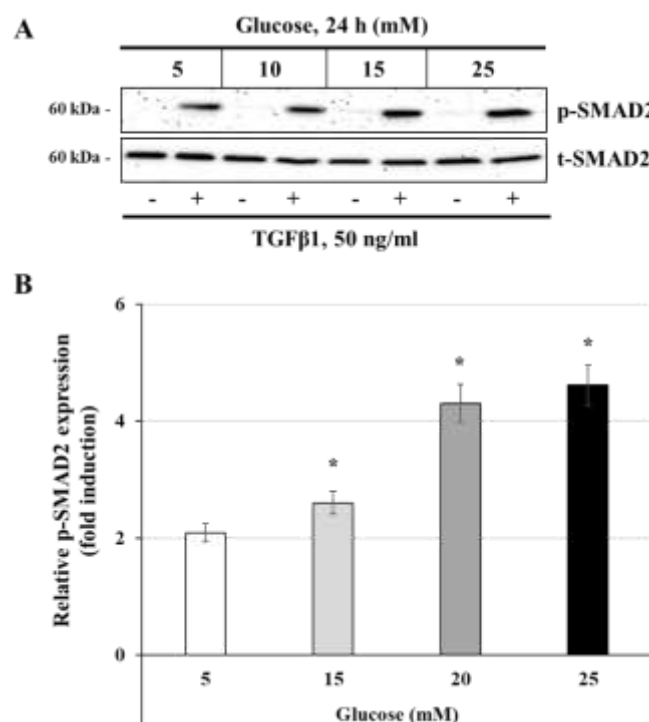


Figure 4. Glucose concentration regulates TGFβ1-induced SMAD2 phosphorylation: (A,B) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (10, 15, and 25 mM), and then stimulated for 30 min with culture media alone (control) or with 50 ng/ml TGFβ1. (A) Representative images showing the expression of phosphorylated SMAD2 (p-SMAD2) and total SMAD2 (t-SMAD2) as evaluated by western blot. (B) Graphical analysis showing the expression levels of p-SMAD2 relative to t-SMAD2 for each glucose concentration (n = 3). * $p < 0.05$ denotes a significant difference compared to control.

3.5. Effect of TGFβ1 on The Process of Glucose And Energy Metabolism

Because high-glucose condition significantly reduced ATP biosynthesis without affecting lactate production, we first examine the influence of glucose concentration and TGFβ1 stimulation in the expression of the glucose transporters GLUT1 and GLUT3 by western blot (Figure 5A). JEG-3 cells were thus cultured for 24 h in media containing 5 mM or 25 mM glucose in the absence (control) or the presence of TGFβ1. Our results indicate that high-glucose condition (25 mM) decrease the expression of both glucose transporters, the relative reduction levels being evaluated at 64 % for GLUT1 (Figure 5B) and 39 % for GLUT3 (Figure 5C) compared to normal-glucose condition. However, TGFβ1 stimulation significantly increases the expression of GLUT1 and GLUT3 at high-glucose concentration, at levels similar to that in normal-glucose condition (Figure 5B,C). Next, we investigate whether TGFβ1 influences lactate and ATP production. As previously shown (Figure 1), our results confirm that JEG-3 cells incubation with normal- or high-glucose concentrations does not affect lactate production (Figure 5D) but incubation with a high-glucose concentration significantly reduced ATP biosynthesis (Figure 5E). Moreover, we found that TGFβ1 does not affect lactate production even after a 24-h stimulation period with TGFβ1 (Figure 5D). However, as shown in figure 5E, treatment with TGFβ1 for 24 h similarly enhances ATP production when cells were incubated with either 5 mM glucose (1.3-fold increase) or 25 mM glucose (1.6-fold increase). Taken together, these results indicate that TGFβ1 appears to influence energy metabolism by targeting mitochondria function rather than lactate production in the glycolytic pathway.

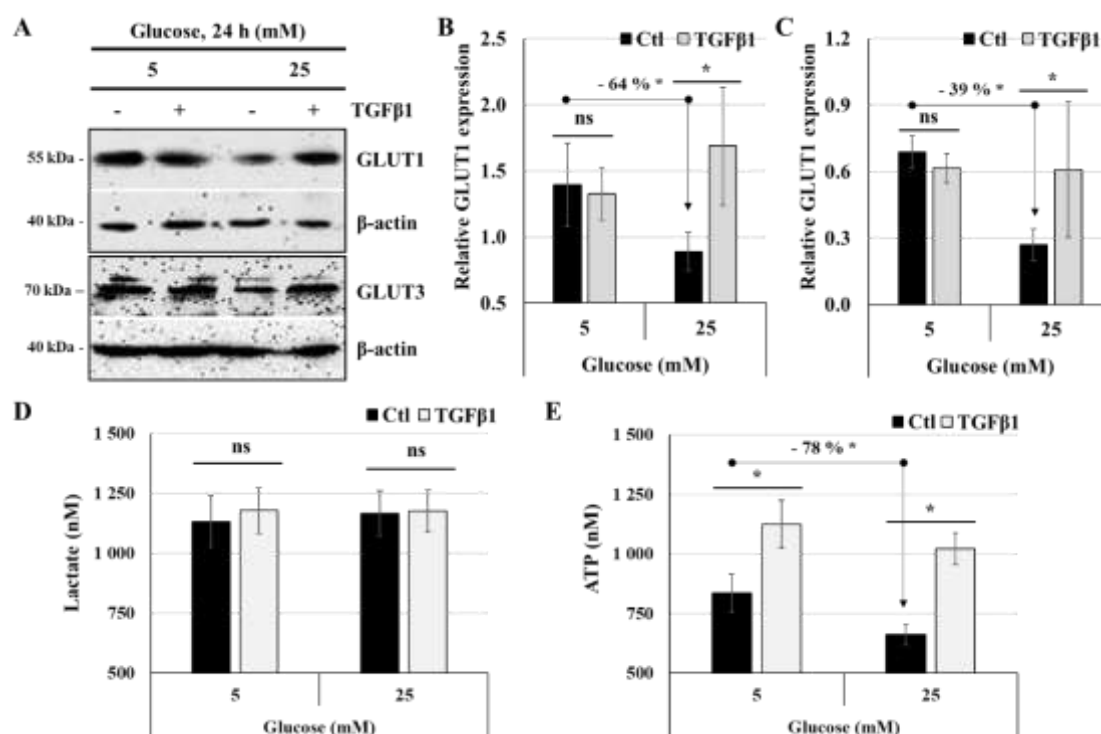


Figure 5. TGFβ1 differentially regulates glucose transporters expression, lactate and ATP production: (A-E) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM) in the absence or the presence of TGFβ1 at 50 ng/ml. (A) Representative images of GLUT1, GLUT3 and b-actin protein detection, as assessed by western blot. Graphical analysis showing the relative expression of GLUT1 (B) and GLUT3 (C) at 5 mM and 25 mM glucose. (D) Quantitation of lactate production was assessed using Lactate-Glo™ assay kit. (E) Quantitation of ATP production was assessed using ATP luminescence detection assay kit. Data are expressed as nM of ATP and lactate (n = 3). Each bar represents the mean ± SD from at least two independent experiments. * $p < 0.05$ indicates a significant difference between the cell groups, and ns = nonsignificant difference.

3.6. Effect of TGFβ1 on Mitochondrial Respiratory Chain Proteins Expression

To better understand the effect of TGFβ1 on the regulation of energy metabolism in JEG-3 cells, we decided to assess the expression of mitochondrial respiratory chain proteins under normal-glucose (5 mM) or high-glucose concentrations (25 mM) in the absence (control) or the presence of TGFβ1 at 50 ng/ml during 24 h. The results showed in figure 6A confirm that exposing JEG-3 cells to high glucose concentrations decreases the expression of mitochondrial respiratory chain protein subunits CI-NDUF88, CII-SDHB, CIII-UQCRC2, and CIV-MTCO1, but results in significant induction of p-SMAD2. Examining Figure 6B more closely, when JEG-3 cells were incubated with 5 mM glucose, treatment with TGFβ1 significantly increased the expression of OXPHOS protein subunits CI-NDUF88 (1.15-fold), CII-SDHB (1.3-fold), CIII-UQCRC2 (1.2-fold), with a more pronounced increase in CIV-MTCO1 (1.5-fold). As shown in figure 6C, when the cells were incubated with a high-glucose concentration (25 mM), TGFβ1 treatment enhanced the expression of OXPHOS protein subunits, with a more pronounced effect on CI-NDUF88 (2.5-fold) and CIII-UQCRC2 (2.6-fold). To better define the influence of TGFβ1 on OXPHOS protein expression at normal- and high-glucose concentrations, we evaluated the mean of total fold induction for each condition and found that TGFβ1 treatment enhanced the expression of these proteins by 1.7-fold when the cells were incubated in media containing 25 mM compared to media containing 5 mM glucose (Figure 6D). Taken together, these results indicate that TGFβ1 treatment not only enhances the mitochondrial respiratory chain under control conditions but also mitigates the damage induced by high-glucose concentration.

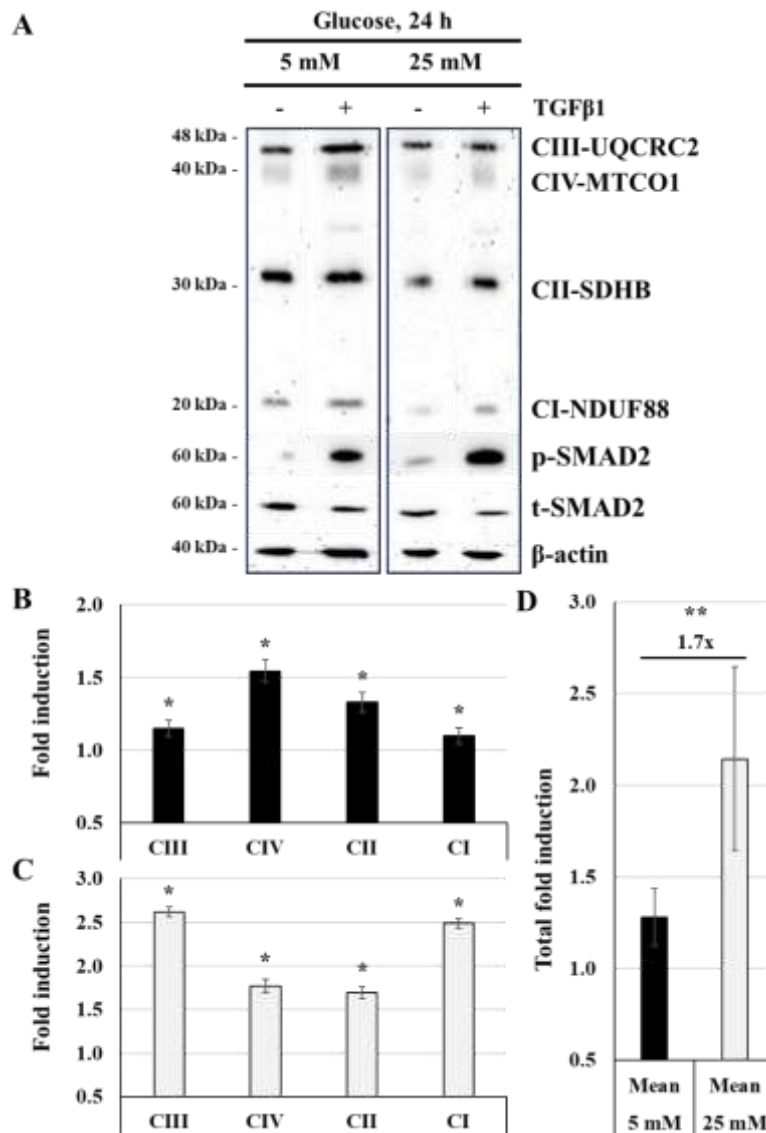


Figure 6. TGFβ1 enhances the expression of the mitochondrial respiratory chain proteins at normal-glucose and high-glucose concentrations: (A,B,C,D) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM) in the absence or the presence of TGFβ1 at 50 ng/ml. (A) Representative images of OXPHOS complexes protein subunits detection, SMAD2 (p-SMAD2 and t-SMAD2), and β-actin, as assessed by western blot. Graphical analysis showing the expression of different mitochondrial complexes proteins subunits relative to β-actin at 5 mM (B) and 25 mM (C) glucose, and the summary of fold induction from all OXPHOS proteins subunits (D). (B,C) * $p < 0.05$ indicates a significant difference compared to control ($n = 3$). (D) ** $p < 0.05$ denotes a significant difference between 5 mM and 25 mM glucose concentration, and ns = nonsignificant difference.

3.7. Effect of TGFβ1 on the Expression of PPARγ

To enhance our understanding of how TGFβ1 affects the energy metabolism in human trophoblast cells, we determined the effect of TGFβ1 on PPARγ expression. JEG-3 cells were cultured for 24 h in media containing 5 mM or 25 mM glucose in the absence or the presence of TGFβ1. Then, PPARγ expression was assessed using western blotting (Figure 7A). Our results demonstrated that cell incubation in high-glucose condition decreases PPARγ expression (relative expression = 1.05 ± 0.08 vs 0.67 ± 0.03 ; Figure 7B) but enhances TGFβ1-induced SMAD2 phosphorylation (Figure 7C), with greater induction at 25 mM glucose (relative expression = 0.83 ± 0.09) compared with 5 mM glucose (relative expression = 0.59 ± 0.09). In normal-glucose condition, treatment with TGFβ1 does not affect PPARγ, as reflected by similar intensity in both normal- and high-glucose concentrations

(Figure 7B). However, TGF β 1 treatment significantly increases PPAR γ expression under high-glucose conditions (relative expression = 1.41 ± 0.09 vs 0.67 ± 0.09 at 5 mM). These results suggest that PPAR γ may play a key role in mediating the function of TGF β 1 on energy homeostasis in JEG-3 cells under high-glucose stressing conditions.

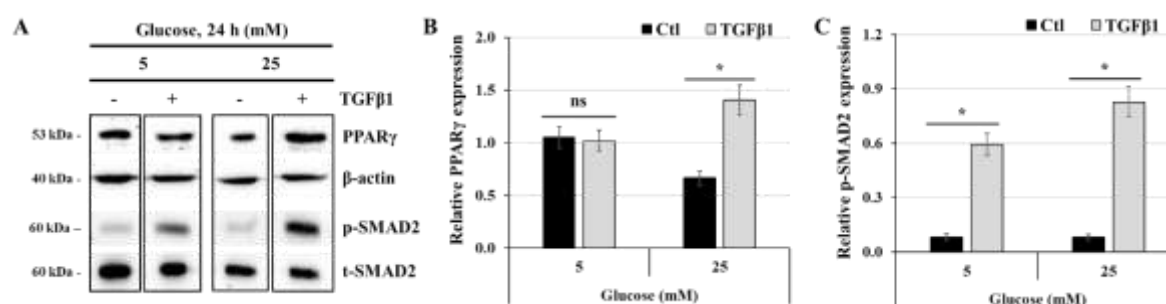


Figure 7. TGF β 1 increases the expression of PPAR γ at high-glucose concentration: (A,B,C) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM) in the absence or the presence of TGF β 1 at 50 ng/ml. (A) Representative images of PPAR γ , β -actin, and SMAD2 (p-SMAD2 and t-SMAD2), as assessed by western blot. Graphical analysis showing the relative expression of PPAR γ (B) and p-SMAD2 (C) at 5 mM and 25 mM glucose (n = 3). * $p < 0.05$ indicates a significant difference between control and TGF β 1 treatments, and ns = nonsignificant difference.

3.8. Effect of TGF β 1 on the Expression of HIF1 α and the Activation of AMPK

To further elucidate the mechanisms of TGF β 1 in the maintenance of ATP balance under normal-glucose and high-glucose stressing conditions, we investigated the regulatory role of TGF β 1 on HIF1 α expression and AMPK activation. In this set of experiments, JEG-3 cells were cultured for 24 h in normal-glucose (5 mM) or high-glucose conditions (25 mM), and then activated in the absence (control) or the presence of TGF β 1 at 50 ng/ml for 15-, 30-, or 60-min (Figure 8A). As expected, treatment with TGF β 1 induces p-SMAD2 with greater phosphorylation levels at 25 mM glucose (relative expression, control = 0.066 ± 0.007 vs TGF β 1 = 0.57 ± 0.08 at $t = 30$ min; Figure 8B) compared with 5 mM glucose (relative expression, control = 0.075 ± 0.008 vs TGF β 1 = 1.062 ± 0.074 at $t = 30$ min; Figure 8B). Noticeable, JEG-3 cells basally express higher levels of HIF1 α protein in both normal- and high-glucose conditions (Figure 8A,C). However, basal levels of HIF1 α expression increases in high-glucose condition (relative expression, 1.07 ± 0.11 vs 0.31 ± 0.02 in 5 mM glucose at $t = 30$ min; Figure 8C), while AMPK activation was unaffected in both normal- and high-glucose conditions (Figure 8D). In contrast, TGF β 1 treatment induces HIF1 α degradation, an effect observed beginning at $t = 15$ min and continuing until the end of the experiment; this effect appears to be dependent on glucose concentration in the media, with greater degradation levels with 25 mM glucose (relative expression, control = 1.17 ± 0.11 vs TGF β 1 = 0.29 ± 0.04 at $t = 60$ min; Figure 8C) compared with 5 mM glucose (relative expression, control = 0.58 ± 0.06 vs TGF β 1 = 0.23 ± 0.02 at $t = 60$ min; Figure 8C). The degradation of HIF1 α was mirrored by the induction of p-AMPK via TGF β 1, and again, with greater phosphorylation levels at 25 mM glucose (relative expression, control = 0.74 ± 0.05 vs TGF β 1 = 2.25 ± 0.13 at $t = 60$ min; Figure 8D) compared with 5 mM glucose (relative expression, control = 0.34 ± 0.03 vs TGF β 1 = 1.34 ± 0.13 at $t = 60$ min; Figure 8D). Taken together, these results indicate that TGF β 1 restores the energy homeostasis of JEG-3 cells under high-glucose stressing conditions by inducing AMPK activation and HIF1 α degradation.

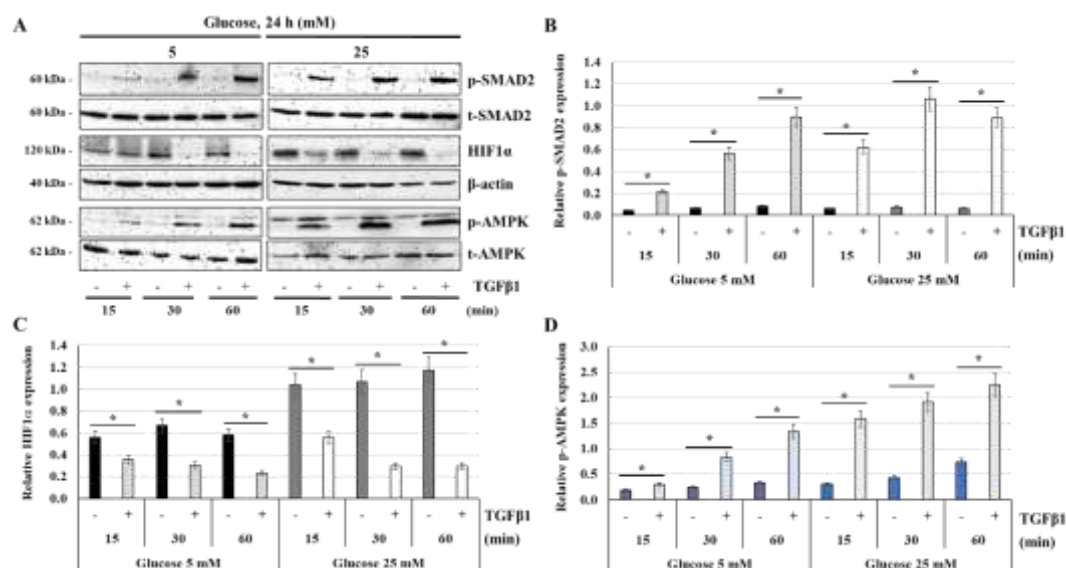


Figure 8. TGFβ1 decreases HIF1α expression but enhances AMPK activation at normal-glucose and high-glucose concentrations: (A,B,C,D) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM), and then activated in the absence or the presence of TGFβ1 at 50 ng/ml for 15, 30, or 60 min. (A) Representative images of SMAD2 (p-SMAD2 and t-SMAD2), HIF1α, β-actin, and AMPK (p-AMPK and t-AMPK), as assessed by western blot. Graphical analysis showing the relative expression of p-SMAD2 (B), HIF1α (C), and p-AMPK (D), at 5 mM and 25 mM glucose (n = 3). $p < 0.05$ indicates a significant difference between the cell groups.

3.9. The Von Hippel-Lindau Protein Antagonist VH298 blocks TGFβ1-induced HIF1α degradation

Under normoxia, HIF1α is maintained at very low levels via proline-hydroxylation by PHD proteins, allowing their recognition and polyubiquitination by the Von Hippel-Lindau (VHL) Cullin RING E3 ubiquitin ligase complex and subsequent proteasomal degradation [17]. To elucidate the mechanisms by which TGFβ1 induces HIF1α degradation, JEG-3 cells were cultured for 24 h in media containing glucose at 5 mM or 25 mM, and then activated for 60 min with TGFβ1 in the absence or the presence of VH298, a potent and specific VHL inhibitor that stabilizes HIF1α by blockade of the VHL:HIF protein-protein interaction downstream of HIF1α hydroxylation by PHD enzymes. As established above (Figure 8), the basal expression levels of HIF1α significantly decreased in both normal- and high-glucose conditions after TGFβ1 stimulation (Figure 9A,B), an effect that was mirrored by enhanced levels of TGFβ1-induced SMAD2 phosphorylation (Figure 8A,C). However, treatment with VH298 molecules induces HIF1α stabilisation with greater expression levels at 25 mM glucose compared with 5 mM glucose (Figure 9A,B). Important to note, the protein expression levels of HIF1α remain stable and unchanged even in the presence of TGFβ1 (Figure 9B). Moreover, the presence of VH298 does not affect the levels of TGFβ1-induced SMAD2 phosphorylation, which were significantly increased in both normal- and high-glucose conditions after TGFβ1 stimulation (Figure 9C). These results suggest that HIF1α protein stabilisation is not caused by a blockade of SMAD2 phosphorylation by VH298 molecules, and that TGFβ1 might induce HIF1α proteasomal degradation via enhanced VHL or PHD enzyme activities.

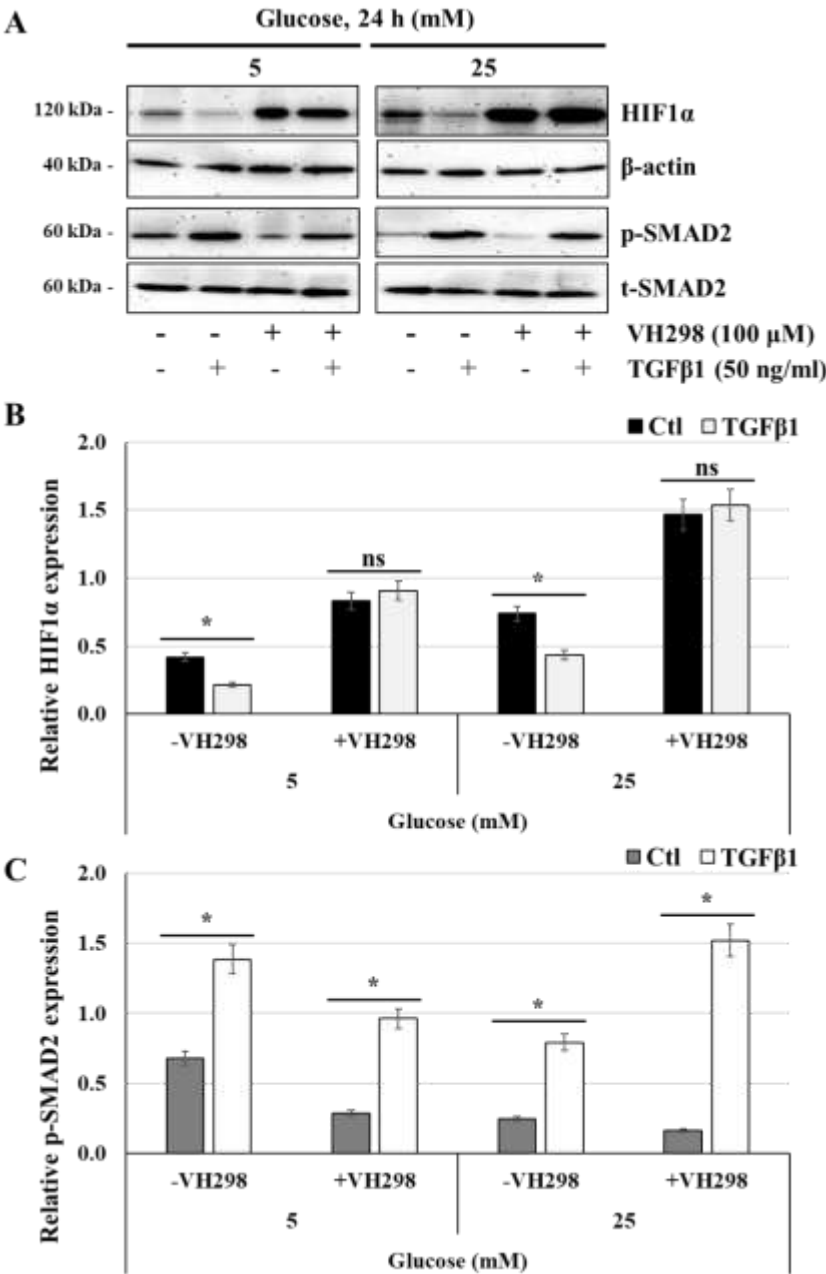


Figure 9. TGFβ1-induced HIF1α degradation is blocked in the presence of VH298, a specific inhibitor of ubiquitylation and proteasomal degradation of HIF1α: (A,B,C) JEG-3 cells were cultured for 24 h in media containing normal-glucose (5 mM) or high-glucose concentrations (25 mM), and then activated for 60 min with TGFβ1 in the absence or the presence of VH298. (A) Representative images of HIF1α, β-actin, and SMAD2 (p-SMAD2 and t-SMAD2), as assessed by western blot. Graphical analysis showing the relative expression of HIF1α (B) and p-SMAD2 (C) at 5 mM and 25 mM glucose (n = 3). * *p* < 0.05 indicates a significant difference between the cell groups, and ns = nonsignificant difference.

4. Discussion

The process of placental transport and metabolism of nutrients essential for fetal development is metabolically expensive and requires abundant oxygen consumption and ATP production, primarily synthesized through glycolysis and oxidative phosphorylation [34]. Glucose is principally the energy source for the placenta and fetus, and all the glucose supplied to the placenta-fetal unit originates from the maternal glucose pool, which is produced by maternal gluconeogenesis or ingested through the pregnant woman's diet [35]. However, elevated glucose levels at the fetal-

maternal interface have been linked to poor maternal and perinatal outcomes. In fact, hyperglycemia is the most common medical condition affecting pregnancy, and its occurrence is rising globally in tandem with the dual epidemics of obesity and diabetes [36]. The potential outcomes of hyperglycemia in pregnancies with diabetes mellitus (DM) and gestational diabetes mellitus (GDM) include preterm birth, preeclampsia, and stillbirth. However, fetal overgrowth and macrosomia are the most common adverse effects [37]. Lipid metabolism is also altered in placentas from DM and GDM women. Thus, it is proposed that maternal hyperglycemia is a contributing factor to fetal macrosomia by enhancing substrate availability to the fetus, and stimulating adipose tissue formation and excessive growth [38].

Hyperglycemia can impair spiral artery remodeling, increasing the risk of pregnancy complications, such as miscarriage, cardiac and renal malformations, and rare neural conditions, such as sacral agenesis [2]. It negatively affects EVT cell function in uterine spiral artery remodeling by disrupting trophoblast proliferation, migration, invasion, hormone and angiogenic factor release, and communication between trophoblasts and immune cells [2].

In this study, we showed that high-glucose concentrations induce alterations in the regulation of energy metabolism in human trophoblast JEG-3 cells, a model of placental EVT cells. Notably, these *in vitro* hyperglycemic conditions reduce ATP synthesis and mitochondrial function without affecting lactate production via the glycolytic pathway. Previous research on trophoblast cells from normal and GDM placentas shows that a high glucose concentration (25 mM) significantly reduces the glycolytic capacity and ATP production of cytotrophoblasts, particularly EVT cells [3]. EVT cells are noted for higher oxygen consumption and glycolysis compared to fusogenic VST cells. Additionally, the mitochondrial respiratory capacity of VST cells from both normal and GDM placentas remains unaffected by glucose level variations. However, VST cells from GDM placentas exhibit significantly lower levels of β -hCG and other syncytialization markers, including GCM1 and syncytin-1, compared to those from normal placentas [3]. According to the results of the microarray transcriptome analysis, DM is associated with alterations in gene expression at critical stages of placental energy metabolism, with 67 % of the changes affecting lipid pathways and 9 % affecting glucose pathways [39]. Furthermore, pregnant women with GDM show preferential activation of lipid genes [39]. Human trophoblast BeWo cells, a model of placental VST cells, were cultured in 5- or 25-mM glucose to identify the functional biochemical pathways perturbed by high glucose levels [40]. Among the pathway networks affected by high-glucose conditions, this study highlighted the phosphoinositide 3-kinase (PI3K) cascade, glucose metabolism, peroxisomal lipid metabolism, phospholipid metabolism, bone morphogenetic protein (BMP)/SMAD2 cascade, regulation of lipid metabolism by peroxisome proliferator-activated receptors (PPARs), and cellular response to stress [40].

Although the precise mechanism by which high glucose concentrations in EVT suppress metabolic activity and mitochondrial oxidative phosphorylation remains largely unknown, our results suggest that these changes may be mediated by reduced expression levels of mitochondrial respiratory chain proteins and PPAR γ , coinciding with increased HIF1 α expression.

Then, because a critical role TGF β 1 play in multiple aspects of cell metabolism [13], we hypothesized that this cytokine regulates energy metabolism in trophoblast cells by influencing the expression of key energy metabolic sensors, such as HIF1 α , PPAR γ , and AMPK, under high-glucose conditions. Our results showed that TGF β 1 stimulation led to increased levels of SMAD2 phosphorylation when the cells were incubated in high-glucose conditions (15 mM and 25 mM). This outcome aligns with prior research demonstrating that prolonged exposure to high glucose levels triggers a range of physiological and pathophysiological changes, activating various signaling pathways that often disrupt the function of cells, tissues, and organ systems [41]. Hyperglycemia can activate protein kinase C (PKC) in malignant cells, leading to the activation of various pathways including Akt, TGF β /SMADs, and NF κ B. These pathways work together to control cancer cell metabolism and behavior such as proliferation, migration, invasion, and recurrence [42]. As research suggests, elevated glucose levels have been found to boost the cell membrane concentrations of T β RI and T β RII, and to promote the activation of latent TGF β by matrix metalloproteinases [13]. This, in

turn, triggers the Akt-mTOR pathway and leads to enlargement of fibroblasts and epithelial cells. Furthermore, TGF β signaling plays a role in regulating other components of the glycolytic pathway [13].

Our findings indicated that TGF β 1 restores the energy homeostasis of JEG-3 cells under high-glucose stress conditions. Specifically, TGF β 1 treatment enhances ATP production and mitochondrial respiratory chain protein expression under high-glucose concentrations. While stressing high-glucose condition does not affect lactate production, even after TGF β 1 stimulation, we found that TGF β 1 restore the expression levels of GLUT1 and GLUT3, at levels like that in normal-glucose conditions. These results are consistent with previous research showing that TGF β 1 improve the activity/expression of GLUT1 and HK2 in different cell types, thus favoring glucose capture and energy production [13]. Moreover, it was established that TGF β 1 is responsible for significant metabolic reprogramming in normal and cancerous cells by promoting mitochondrial ATP production [14], and increasing mitochondrial activity, as evidenced by the higher mitochondrial DNA copy number and ROS levels [43]. Furthermore, TGF β 1 activates the AMPK pathway, which promotes fatty acid oxidation and inhibits anabolic pathways [44]. It is proposed that this effect on mitochondrial metabolism can help normal and cancer cells maintain their energy requirements and metabolic adaptations through metabolic stressing conditions.

The impact of TGF β 1 on trophoblast metabolism is not extensively investigated. Here, we demonstrated that TGF β 1 treatment of trophoblast JEG-3 cells resulted in the degradation of HIF1 α under normal- and high-glucose concentrations. Although prior studies have demonstrated that TGF β 1 induces HIF1 α stability under hypoxic and normoxic conditions by inhibiting the prolyl hydroxylase domain protein PHD2, an oxygen sensor that typically promotes HIF1 α degradation [45]. Noticeable, the stability and function of HIF1 α is also regulated by hyperglycemia through interference with the degradation of HIF1 α triggered by PHD enzymes [46]. The findings suggest the presence of dual effects of TGF β 1 on HIF1 α stability and degradation in different physiological and pathological contexts. In this study, we demonstrated that TGF β 1-induced HIF1 α degradation is blocked in the presence of VH298, a potent and specific VHL inhibitor that disrupt VHL: HIF protein-protein interaction and subsequent HIF1 α proteasomal degradation, suggesting that TGF β 1 might induce HIF1 α proteasomal degradation via enhanced VHL or PHD enzyme activities. Nonetheless, additional research is necessary to understand the precise mechanism by which TGF β 1 causes HIF1 α degradation in JEG-3 cells.

The impact of TGF β 1 on HIF1 α in JEG-3 cells was accompanied by the activation of AMPK, regardless of glucose concentration. This outcome aligns with research suggesting that TGF β 1 can indirectly activate AMPK through various signaling pathways [47]. Particularly, IGF-I and IGFBP-2 have been shown to stimulate AMPK activation and assisting in modulating AMPK activity that plays a vital role in maintaining energy balance and metabolic regulation [47].

Conversely, our findings demonstrated that glucose concentration affected the effect of TGF β 1 on PPAR γ . Specifically, we found that TGF β 1 did not affect PPAR γ expression under normal glucose levels. These results indicate a potential interaction between PPAR γ and AMPK in relation to the effect of TGF β 1 on ATP production. AMPK activation enhances the expression of genes related to fatty acid oxidation (FAO) through interaction with PPAR γ , thereby improving mitochondrial function and ATP production. Independent research indicates that combined activation of AMPK and PPAR γ synergistically upregulates FAO genes, boosting ATP levels in macrophages [48, 49]. The AMPK-PPAR γ coactivator 1 α (PGC-1 α) pathway is crucial for mitochondrial biogenesis and function, as it enhances PPAR γ activity, thereby increasing the expression of mitochondrial genes and promoting ATP production. This pathway is vital for maintaining energy homeostasis in macrophages under metabolic stress [48, 49].

Our results indicate that TGF β 1 restores PPAR γ expression under high glucose concentrations, suggesting that PPAR γ is involved in enhancing ATP production in JEG-3 cells under high glucose stress. Even though earlier investigations have revealed that the activation of PPAR γ by agonists in β -cells can promote mitochondrial energy metabolism and improve ATP production. This regulation helps manage glucose-stimulated insulin secretion under lipotoxic conditions [50, 51]. Investigations

have revealed that activation of PPAR γ in prostate cancer cells increased mitochondrial biogenesis and ATP levels by upregulating AKT3, which enhanced the nuclear localization of PGC1 α [50, 51].

5. Conclusions

Elevated blood glucose levels at the fetal-maternal interface are associated with problems in placental trophoblasts and an increased risk of pregnancy-related complications. Our study suggests that TGF β 1 may play a protective role against the negative effects of high-glucose concentrations on trophoblast JEG-3 cells. Our results showed that high glucose treatments disrupted energy and mitochondrial metabolism, which was indicated by a decrease in ATP production without affecting lactate production. At the molecular level, this was demonstrated by an increase in HIF1 α expression and nuclear localization, a decrease in PPAR γ expression and nuclear localization, and a decrease in the expression of GLUT1 and GLUT3, and respiratory chain proteins. Our study is the first to demonstrate that TGF β 1 can have a protective effect by restoring ATP production under high-glucose stress conditions by inducing HIF1 α degradation, AMPK activation, and restoring PPAR γ and respiratory chain protein expression (Figure 10).

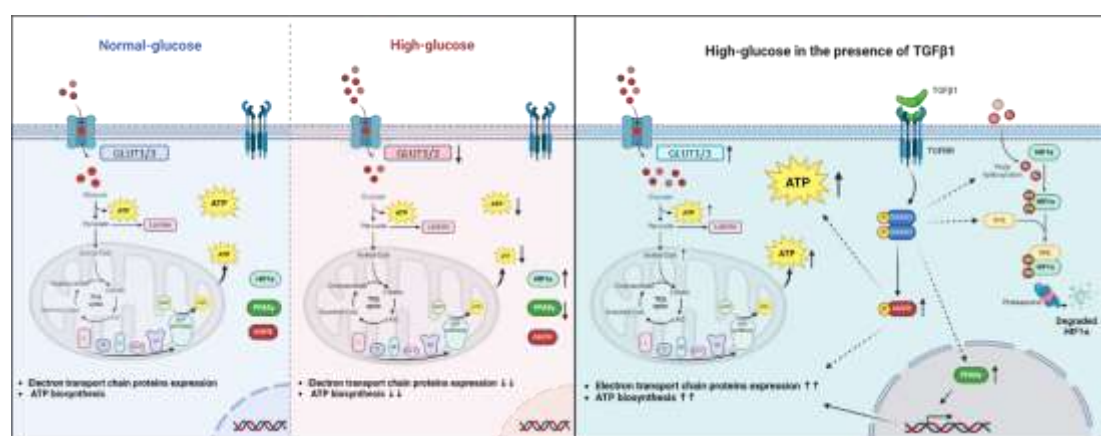


Figure 10. Graphical representation suggesting a protective effect of TGF β 1/SMAD signaling pathways in trophoblast cells under high-glucose stress conditions by restoring ATP production via induction of HIF1 α degradation, AMPK activation, and restoring PPAR γ , GLUT1/3, and electron transport chain protein expression.

These results are significant because they add to the list of TGF β 1's effects during pregnancy. TGF β 1 is essential for successful pregnancy by regulating immune tolerance, trophoblast invasion, and placental development. Dysregulation of TGF β 1 signaling, as observed in preeclampsia and intrauterine growth restriction, can lead to severe complications, emphasizing its crucial role in maintaining pregnancy health. However, further research is needed to better understand the role of TGF β 1 in regulating glucose metabolism and energy balance in the placenta and in trophoblast cells and its potential use in protection against gestational complications.

The trophoblast JEG-3 cell is a choriocarcinoma cell line, which presents a limitation in the study design that impact the ability to interpret and generalize from our research results. In fact, due to ethical restrictions and limited availability of human tissues, trophoblast cell lines are generally used to investigate many aspects of trophoblast biology and metabolism. According to a study by Lee et al. (2016), JEG-3 cells share some expression characteristics with primary human trophoblast cells, particularly the expression of C19MC miRNAs [52]. Additionally, KRT7, GATA3, and TFAP2C are emphasized as reliable markers for identifying primary trophoblasts, supporting the characterization of JEG-3 cells. Additionally, a study by Weiss et al. (2001) showed that compared to other immortalized cell lines, JEG-3 cells showed higher expression of glucose transporters GLUT1 and GLUT3, suggesting a greater capacity for glucose uptake [53]. Future *in vitro* investigations using human trophoblast stem cells or placental explants will further provide us insight into fundamental

biological effects and the regulating role of TGF β 1 in different metabolic conditions. Moreover, we also plan to confirm whether these TGF β 1 mechanisms operate in an *in vivo* model.

Author Contributions: Conceptualization, C.R.-M.; methodology, C.R.-M. and N.K.; validation, N.K, J.G.; formal analysis, C.R.-M. and N.K.; investigation, N.K, J.G and E.S.K.T. ; resources, J.G.; writing—original draft preparation, N.K. and C.R.-M.; writing—review and editing, C.R.M., supervision, C.R.-M., C.V., C.V.T.; project administration, C.R.-M.; funding acquisition, C.R.-M., C.V., C.V.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; number RGPIN/6516-2014 to C.R.-M.; number RGPIN-386602-2011 to C.V.T.; and number RGPIN/06778-2019 to C.V.); the Fonds Québécois de la Recherche sur la Nature et les Technologies-Réseau Québécois en Reproduction (FQRNT-RQR; number 2018-RS4-203214 to C.R.-M. and C.V.); the FQRNT-RQR scholarships program supported K.N., and E.S.K.T. holds undergraduate and graduate scholarships from the NSERC and the RQR-CREATE scholarships programs, respectively.

Institutional Review Board Statement: The human placental choriocarcinoma JEG-3 cell line (#HTB-36) was obtained from ATCC (Rockville, MD, USA).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: We would like to thank the Réseau Québécois en Reproduction (RQR) and the club avenir foundation for their financial support that helped realize this project. We thank Ms. Marion Ravelojaona for her help in producing the graphical abstract.

Conflicts of Interest: The authors declare no conflicts of interest regarding the publication of this paper.

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