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ORIGINAL ARTICLE

Hexosamine pathway regulates StarD7 expression in JEG-3 cells

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Abstract

StarD7 is a lipid binding protein involved in the delivery of phosphatidylcholine to the mitochondria whose promoter is activated by Wnt/β-catenin signaling. Although the majority of glucose enters glycolysis, ~2–5% of it can be metabolized via the hexosamine biosynthetic pathway (HBP). Considering that HBP has been implicated in the regulation of β-catenin we explored if changes in glucose levels modulate StarD7 expression by the HBP in trophoblast cells. We found an increase in StarD7 as well as in β-catenin expression following high-glucose (25 mM) treatment in JEG-3 cells; these effects were abolished in the presence of HBP inhibitors. Moreover, since HBP is able to promote unfolded protein response (UPR) the protein levels of GRP78, Ire1α, calnexin, p-eIF2α and total eIF2α as well as XBP1 mRNA was measured. Our results indicate that a diminution in glucose concentration leads to a decrease in StarD7 expression and an increase in the UPR markers: GRP78 and Ire1α. Conversely, an increase in glucose is associated to high StarD7 levels and low GRP78 expression, phospho-eIF2α and XBP1 splicing, although Ire1α remains high when cells are restored to high glucose. Taken together these findings indicate that glucose modulates StarD7 and β-catenin expression through the HBP associated to UPR, suggesting the existence of a link between UPR and HBP in trophoblast cells. This is the first study reporting the effects of glucose on StarD7 in trophoblast cells. These data highlight the importance to explore the role of StarD7 in placenta disorders related to nutrient availability.

Keywords Hexosamine pathway · StarD7 · START domain · JEG-3 cells · UPR

Abbreviations

AZA	*O*-Diazoacetyl-L-serine
DON	6-Diazo-5-oxo-L-norleucine
eIF2α	Eukaryotic translation initiation factor 2 subunit 1α
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GFAT	Glutamine fructose-6-phosphate amidotransferase
GlcNAc	*N*-acetylglucosamine
GRP78	Glucose regulated protein 78
HBP	Hexosamine biosynthetic pathway
IRE1α	Inositol-requiring enzyme 1
O-GlcNAc	O-GlcNAcylation
OGT	O-GlcNAc transferase

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siRNA	Small interfering RNA
StarD7	StAR-related lipid transfer (START) domain containing 7
TBS	Tris buffered saline
UDP-GlcNAc	Uridine diphosphate *N*-acetylglucosamine
UPR	Unfolding protein response.

Introduction

Mammalian cells adapt to fluctuations in nutrient supply and energy metabolism through complex molecular mechanisms [1]. Among them the hexosamine biosynthetic pathway (HBP) senses the nutritional condition of the cell and uses the glycolytic derivatives to produce uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) [2, 3]. This compound participates in several glycosylation reactions such as N-glycosylation, O-glycosylation and O-GlcNAcylation (O-GlcNAc) [2]. Glucose flux through the HBP leads to modification of various intracellular proteins with O-linked GlcNAc [4]. O-GlcNAc is the covalent attachment of *N*-acetylglucosamine (GlcNAc) sugar to serine or threonine residues of proteins. O-GlcNAc controls the activity of a large number of cytoplasmic, nuclear, and mitochondrial proteins impacting in a variety of cellular processes such as epigenetic, cytokinesis, transcription, translation, protein degradation, metabolism, and ribosomal bioenergetics [3, 5]. A convergence of HBP and endoplasmic reticulum (ER) stress signals leading to the initiation of unfolded protein response (UPR) has been reported in skeletal muscle [6] and HepG2 cells [7], however no data is available in trophoblasts. Cells activate the UPR via three ER transmembrane sensors: inositol requiring kinase 1α (Ire1α), activating transcription factor-6 (ATF6), and protein kinase-like ER kinase (PERK) which interact with the ER luminal GRP78 in resting conditions [8]. The accumulation of unfolded proteins in the ER promotes GRP78 releasing from each sensor which in turn activates the expression of several proteins such as XBP1, ATF4, p50ATF6 and CHOP to reestablish cellular homeostasis. Additionally, PERK pathway leads to the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) that inhibits general mRNA translation. If the cell cannot alleviate the ER stress apoptosis is activated.

StarD7 is a lipid binding protein whose mRNA was initially identified in the choriocarcinoma-derived JEG-3 cells [9]. StarD7 is able to bind and interact with lipids, mainly to transfer phosphatidylcholine (PC) to the mitochondria [10–15]. In addition, StarD7 is involved in trophoblast cells physiology [16], as well as in maintaining ER and mitochondria morphology [17–19]. Furthermore, the viability of StarD7 knockout mouse embryos is severely decreased, underscoring a significant role of this protein in development [20].

Since several reports indicate that glucose through the HBP is involved in the regulation of β-catenin [21–25] and considering that StarD7 gene promoter is activated by Wnt/β-catenin signaling [26] we hypothesize that changes in glucose levels modulate StarD7 expression by the HBP pathway in JEG-3 derived-trophoblast cells.

The findings of this study demonstrate that high glucose regulates StarD7 and β-catenin expression through the HBP associated to a modulation of the UPR signaling, suggesting the existence of a link between UPR and HBP in trophoblast cells.

Materials and methods

Antibodies

Rabbit monoclonal anti-phospho-eIF2α (Ser51) (3398) and rabbit polyclonal anti-total-eIF2α (9722) were from Cell Signaling Technology. Mouse monoclonal anti-α-tubulin (Clone B-5-1-2) was obtained from Sigma Chemical Co. Anti-StarD7Ct was generated in our laboratory as previously described [10]. All antibodies against ER stress markers (ER Stress Antibody Sampler Kit #9956) were from Cell Signaling Technology. IRDye 800CW donkey anti-rabbit IgG (P/N 926-32213) and IRDye 680RD donkey anti-mouse IgG (P/N 926-68073) were obtained from Li-Cor Biosciences.

Cell culture

The human choriocarcinoma cell line JEG-3 (ATCC, HTB-36) was purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM high glucose, ThermoFisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 µg/ml penicillin, 100 µg/ml streptomycin (Thermo Fisher Scientific). Cells were harvested, seeded in 6-well plates at 6×10^5 cells/well, and incubated overnight in serum-free DMEM (no glucose, Thermo Fisher Scientific) containing 0.5 mM glucose at 37 °C and 5% CO_2. After that, cells were changed to DMEM (no glucose) 10% (v/v) FBS, supplemented with either 0.5 mM glucose plus 24.5 mM mannitol, or 5.5 mM glucose plus 19.5 mM mannitol, or 25 mM glucose, and cultured for different time periods. Alternatively, cells grown in DMEM high glucose (25 mM glucose) 10% (v/v) FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin were transferred to 6-well plates at 6×10^5 cells/well and incubated in DMEM (no glucose) with or without 10% (v/v) FBS containing 0.5 mM glucose plus 24.5 mM mannitol, or 5.5 mM glucose plus 19.5 mM mannitol, or they were maintained in high glucose (25 mM with serum) for 24 h. Additionally, cells were cultured in DMEM (no glucose) 10% (v/v) FBS, containing 25 mM of glucose

and 6-Diazo-5-oxo-L-norleucine (DON) or *O*-Diazoacetyl-L-serine (AZA) (Sigma Chemical Co), at the indicated concentrations, in the presence or not of 10 mM glucosamine ($GlcNH_2$) for 24 h.

SDS-PAGE and western blotting

Protein samples were loaded onto 10% SDS-PAGE gels. After migration, proteins were electrotransferred to nitrocellulose (Amersham Bioscience). The membrane was blocked in Tris buffered saline (TBS) (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.2% (v/v) Tween-20 and 5% (w/v) non-fat dry milk, washed and incubated with each one of the following primary antibodies: anti-StarD7Ct (0.5 µg/ml), anti-α-tubulin (1:3000), anti-β catenin (1:1000), anti-total-eIF2α (1:500), and anti-p-eIF2α (1:500), for 1 h at room temperature or overnight 4 °C with shaking, as indicated by the manufactures. The blots for ER stress markers were analyzed with the ER Stress Antibody Sampler Kit (Cell Signaling Technology) according to the manufacturer's instructions. After washing, the blots were incubated with IRDye 800CW donkey anti-rabbit IG or IRDye 680RD donkey anti-mouse IgG antibodies (1:15,000) in TBS for 1 h, protected from light. After washing with TBS plus 0.2% (v/v) Tween-20, the membranes were visualized and quantified using the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA). Protein expression was normalized to the α-tubulin expression.

Analysis of XBP1 mRNA splicing by real time PCR (qPCR)

Total RNA was extracted from cultured cells using Trizol (Invitrogen), according to the manufacturer's instructions. Single-stranded cDNAs were synthesized with random primers (Invitrogen) in 20 µl final volume. Briefly, 1 µg of total RNA was incubated with random primers (1.25 ng/µl) and the reverse transcriptase reaction was performed as previously described [9].

To evaluate XBP1 activation, the splicing of XBP1 mRNA was examined by qPCR according to van Schadewijk et al. [27]. Briefly, cDNA was mixed with SYBR Green PCR Master Mix (Applied Biosystems) and the forward: 5′-TGC TGAGTCCGCAGCAGGTG-3′and reverse: 5′-CTGGCA GGCTCTGGGGAAG-3′ primers were added to a final volume of 20 µl. qPCR was carried out on an Applied Biosystems 7500 Real-Time PCR System with Sequence Detection Software v1.4. The cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Each sample was analyzed in triplicate. Transcript levels were normalized to those of cyclophilin A and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [28]. Amplification efficiency for each set of primers was near 98%. PCR reactions using water instead of template showed no amplification.

Data analysis

Significant differences for control and test conditions were identified using the nonparametric paired Wilcoxon test or unpaired Mann–Whitney *U* test. A Kruskal–Wallis with a Dunns post-test was performed to obtain a multiple comparison of independent sample populations. Significance was taken as $p < 0.01$.

Results

Changes in glucose concentration modify StarD7 and β-catenin expression

Based on the evidence that glucose concentrations regulate β-catenin protein levels [25] and considering that we previously demonstrated that β-catenin induces StarD7 gene promoter [26] we explored the effects of glucose on StarD7 expression. First, StarD7 expression level was evaluated in JEG-3 cells starved during 24 h in DMEM (no glucose) culture medium supplemented with 5.5 or 0.5 mM glucose with or without serum. Western blotting revealed a significant decrease in StarD7 protein expression in cells cultured in medium containing 0.5 mM glucose with or without serum compared to cells maintained in medium with 25 mM glucose and serum (Fig. 1a). Downregulation of StarD7 by glucose deprivation was observed even in the presence of serum, indicating that downregulation of StarD7 levels were due to changes in glucose concentration and not to serum deprivation. Subsequently, JEG-3 cells were cultured overnight in DMEM (no glucose) with 0.5 mM glucose without serum and then they were shifted to a media containing different glucose concentrations (0.5, 5.5, and 25 mM) and serum for up to 24 h. Results indicated that glucose addition results in an early increase (2 h) in StarD7 protein expression level, which was significant at 24 h of incubation in media containing 5.5 or 25 mM glucose (Fig. 1b). Notably, glucose modulation of StarD7 expression paralleled that of β-catenin protein levels (Fig. 1b).

HBP is involved in the effect of glucose on StarD7 expression

To gain insight into the underlying mechanism of how high glucose levels induce StarD7 expression and since it is well documented that the HBP is involved in the regulation of β-catenin by glucose [23, 25], we explored whether HBP is implicated in the effect of glucose on StarD7 expression.

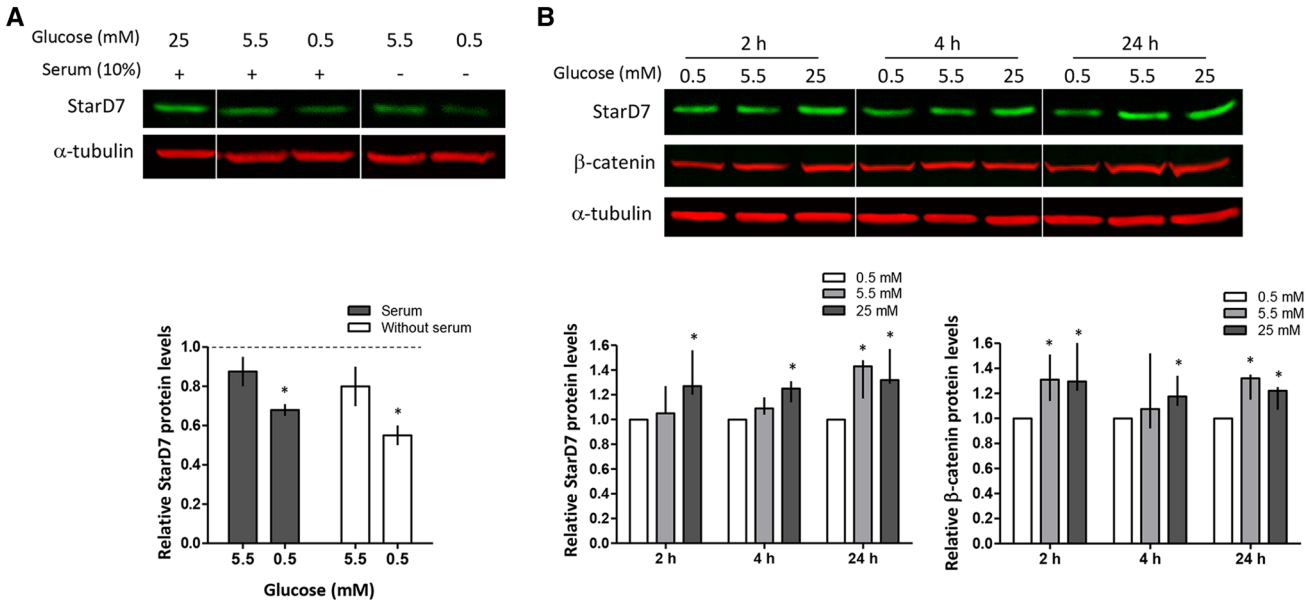

Fig. 1 Changes in glucose concentration modify StarD7 and β-catenin expression. **a** Western blot analysis of StarD7 in protein extracts from JEG-3 cells cultured in 25 mM glucose and serum or in 5.5 mM or 0.5 mM glucose with or without serum for 24 h. The graph represents the densitometric analysis of the StarD7 expression level normalized to α-tubulin expression of at least three independent experiments, and expressed relative to the corresponding protein level determined in 25 mM glucose and serum defined as 1. **b** Western blots analysis of StarD7 and β-catenin in protein extracts from JEG-3 cells previously starved and then cultured with different glucose concentrations for up to 24 h, as indicated in material and methods. α-tubulin was used as a loading control. The graphs represent the densitometric analysis of the expression level of the indicated proteins in JEG-3 cells treated with 5.5 and 25 mM glucose normalized to α-tubulin expression of at least three independent experiments, and expressed relative to the normalized level in cells treated with 0.5 mM glucose defined as 1. Values are median and 25th–75th percentiles of three experiments. *Statistically significant difference from control ($p < 0.01$)

For this purpose, two inhibitors of the rate limiting enzyme: glutamine fructose-6-phosphate amidotransferase (GFAT), DON and AZA, were used (Fig. 2a). JEG-3 cells were cultured in media containing 25 mM of glucose, serum and 0, 10, 50, or 100 μM inhibitors for 24 h. As expected, a dose-dependent decrease in the protein level of β-catenin following DON and AZA treatment was observed. Similarly, a diminution in StarD7 protein level was determined after GFAT inhibition. To further demonstrate the involvement of the HBP in StarD7 expression, $GlcNH_2$, which can directly enter the HBP downstream of GFAT, was added to the cell culture medium. The addition of 10 mM $GlcNH_2$ to DON or AZA treatment (100 μM) partially rescues the effects of GFAT inhibitors, confirming that the effect of glucose on StarD7 expression is conducted by the HBP (Fig. 2b).

Glucose modulates UPR signaling in JEG-3 cells

Next, we explored whether changes in glucose concentration triggered ER stress response /UPR signaling in JEG-3 cells. The levels of GRP78, Ire1α, calnexin, p-eIF2α and total eIF2α were assessed by western blot analysis whereas mRNA of sXBP1 was measured by qPCR. GRP78 expression levels, the classical marker of the UPR, were significantly raised after 24 h of starvation (~ 14-fold, $p < 0.01$) whereas the Ire1α protein was elevated 1.5-fold compared to cells maintained in serum and 25 mM glucose (Fig. 3a). Moreover, an early induction of GRP78 levels was observed (2 h) when cells were moved to a media containing 5.5 or 25 mM glucose plus serum respect to those maintained in 0.5 mM glucose plus serum (Fig. 3b, c). However, GRP78 levels decreased in the presence of 5.5 and 25 mM glucose after 24 h. The GRP78 levels in these last conditions were similar to those measured in cells cultured with serum and 25 mM glucose (see Fig. 3a). A significant increase in the expression of Ire1α protein was detected at 2 and 24 h after the addition of 25 mM glucose; whereas no change was observed in calnexin protein expression. Unexpectedly, the phosphorylation of eIF2α at Ser 51, a well-known ER stress marker, decreased in cells exposed to increased levels of glucose concentration in all the time points assayed (Fig. 3b, c). The sXBP1 mRNA level was higher in cells exposed to 5.5 or 25 mM glucose concentrations at 2 and 4 h of culture, and its abundance was decreased when cells were cultured for 24 h (Fig. 3d). The changes in sXBP1 mRNA are in line with the changes of GRP78 levels detected at the same culture conditions.

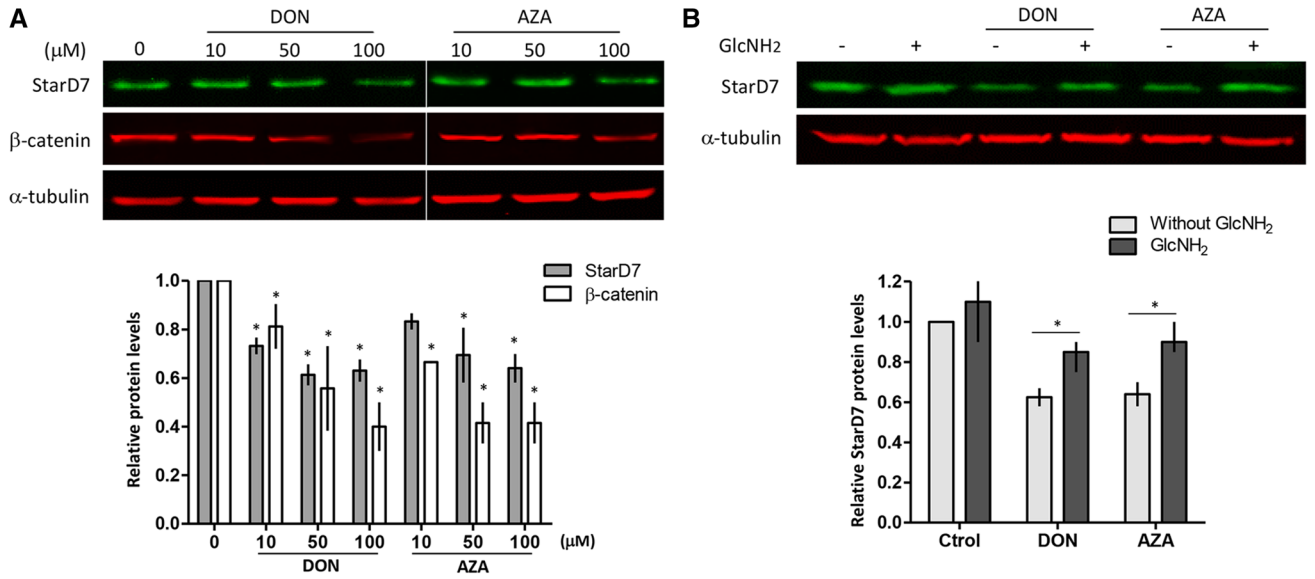

Fig. 2 HBP is involved in the effect of glucose on StarD7 expression. **a** Western blot analysis of StarD7 and β-catenin in protein extracts from JEG-3 cells cultured in 25 mM glucose and serum without or with DON or AZA, at the indicated concentrations, for 24 h. α-tubulin was used as a loading control. **b** Western blot analysis of StarD7 in protein extracts from JEG-3 cells cultured in 25 mM glucose and serum without or with 100 μM of DON or AZA, in the presence or not of 10 mM $GlcNH_2$ for 24 h. The graphs represent the densitometric analysis of the expression level of the indicated proteins in JEG-3 cells normalized to α-tubulin expression of at least three independent experiments, and expressed relative to the corresponding normalized level in non-treated cells defined as 1. Values are median and 25th–75th percentiles of three experiments. *Statistically significant difference from control ($p < 0.01$)

Collectively these data indicate that StarD7 expression is modulated by glucose concentration through the HBP. In addition, StarD7 expression is reduced upon starvation connected with an increase in GRP78 and Ire1α protein levels suggesting an activation of the UPR. Conversely, StarD7 levels are upregulated upon restoration of cells to a culture media with high glucose for 24 h, coincident with a downregulation of GRP78 levels, XBP1 mRNA splicing and phosphorylation of eIF2α at Ser 51. Therefore, present results support the notion of a link among StarD7, HBP and UPR in trophoblasts.

Discussion

HBP nutrient-sensing metabolic pathway leads to an increase in amino sugar UDP-GlcNAc, a critical substrate for protein glycosylation including the O-GlcNAc [29, 30]. HBP was proposed as a nutrient sensor in human placenta during the first-trimester modulating hormone production and IGF signaling [31]. In addition, O-GlcNAc transferase (OGT) enzyme appears as an important placental biomarker of maternal stress [32–34]. A recent study demonstrates that mTOR suppression induces autophagy in HTR8/SVneo cells through the modulation of Beclin1 and SNAP29 O-GlcNAc [35]. Herein, we demonstrated for the first time the effect of glucose concentration on StarD7 protein levels through HBP together with UPR modulation in the trophoblast-derived JEG-3 cells. We established an increase in StarD7 protein amounts in response to elevated glucose. This increase was accompanied with a higher amount of β-catenin. Additionally, we clearly showed that the inhibition of HBP decreases the level of β-catenin and StarD7 expression in high glucose. Moreover, the inhibitory effect of DON or AZA on StarD7 expression was rescued by the addition of $GlcNH_2$ confirming the involvement of HBP in the regulation of StarD7 expression.

The regulation of β-catenin via HBP in this cell line is consistent with several studies that already pointed out the crucial role of HBP in the expression and localization of β-catenin in different cell lines [21–25, 36]. The regulation of StarD7 expression by glucose could be mediated by the upregulation of β-catenin. Accordingly, we have previously shown that StarD7 expression is directly activated by binding of β-catenin to its promoter region in JEG-3 cells [26, 37]. Alternatively, StarD7 expression could also be regulated at the post-translational level through its modification by O-linked GlcNac. In this regard, StarD7 was identified as a putative interactor of OGT by a human proteome microarray and the analysis of the OGT interactome [38].

It is well-known that O-GlcNAc protein modifications is an additional mechanism by which cells sense and respond to different kind of stress stimuli as part of pro-survival signaling [30, 39–41]. In this study we demonstrated that

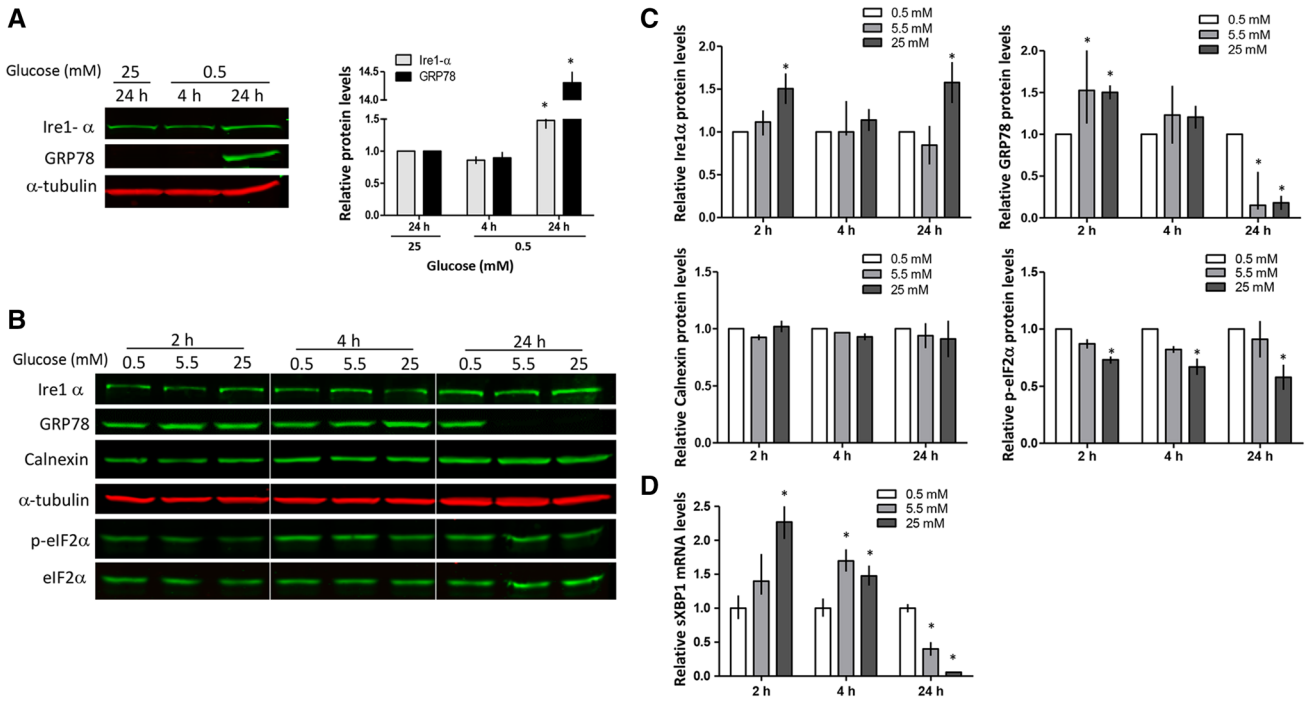

Fig. 3 Glucose modulates UPR signaling in JEG-3 cells. **a** Western blot analysis of GRP78 and Ire1α in protein extracts from JEG-3 cells cultured in starvation conditions (0.5 mM glucose without serum) or in 25 mM glucose and serum for up to 24 h. The graph represents the densitometric analysis of the expression level of the indicated proteins in JEG-3 cells normalized to α-tubulin expression of at least three independent experiments, and expressed relative to the corresponding protein level determined in non-starvation conditions defined as 1. **b** Western blot analysis of GRP78, Ire1α, calnexin, p-eIF2α, and total eIF2α in protein extracts from JEG-3 cells starved overnight (0.5 mM glucose without serum) and then cultured with different glucose concentrations for up to 24 h, as indicated. α-tubulin was used as a loading control. **c** The graphs represent the densitometric analysis of the expression level of the indicated proteins in JEG-3 cells treated with 5.5 and 25 mM glucose normalized to α-tubulin expression of at least three independent experiments, and expressed relative to the corresponding normalized level in cells treated with 0.5 mM glucose defined as 1. Values are median and 25th–75th percentiles of three experiments. **d** sXBP1 mRNA assayed by qPCR from JEG-3 cells exposed to 0.5, 5.5 or 25 mM glucose during 2, 4, or 24 h. *Statistically significant difference from control ($p < 0.01$)

changes in glucose concentrations modulate not only StarD7 expression but also ER stress in JEG-3 cells regulating GRP78 and Ire1α protein levels, XBP1 splicing and eIF2α phosphorylation. A diminution in glucose concentration leads to a decrease in StarD7 expression and an increase in the UPR markers: GRP78 and Ire1α. Conversely, an increase in glucose is associated to high StarD7 levels and low GRP78 expression, phospho-eIF2α and XBP1 splicing, although Ire1α remains high when cells are restored to high glucose for 24 h. We have previously demonstrated that a downregulation of StarD7 by siRNA leads to an early ER stress response [17]. Thus, a reduction in glucose levels may activate the UPR in JEG-3 cells in part through a downregulation of StarD7 protein levels. The phosphorylation of eIF2α at Ser 51, a well-known ER stress marker, was decreased during the first 4 h of incubation in cells exposed to increased levels of glucose concentration when clearly UPR signaling was activated. This finding was probably due to a competition between O-GlcNAc and phosphorylation for the protein substrate. This result is in accordance with the data reported by Jang et al. who demonstrated that O-GlcNAc of eIF2α regulates the phospho-eIF2α-mediated ER stress response [42].

In summary, this study shows that changes in glucose concentration modulate StarD7 expression through HBP along with UPR signaling in trophoblast cells.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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