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Expression of proliferation related transcription factor genes in U87 glioma cells with IRE1 knockdown: upon glucose and glutamine deprivation

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Glycolysis and glutaminolysis as well as endoplasmic reticulum stress are required for tumor progression suggests through regulation of the cell cycle. Inhibition of ERN1/IRE1 (endoplasmic reticulum to nucleus signaling 1/inositol requiring enzyme 1), a central mediator of endoplasmic reticulum stress, significantly suppresses glioma cell proliferation and tumor growth as well as modifies sensitivity gene expressions to glucose and glutamine deprivation. We have studied the expression of genes encoded transcription factors such as E2F8 (E2F transcription factor 8), EPAS1 (endothelial PAS domain protein 1), HOXC6 (homeobox C6), TBX3 (T-box 3), TBX2 (T-box 2), GTF2F2 (general transcription factor IIF), GTF2B (general transcription factor IIB), MAZ (MYC-associated zinc finger protein, purine-binding transcription factor), SNAI2 (snail family zinc finger 2), TCF3 (transcription factor 3), and TCF8/ZEB1 (zinc finger E-box binding homeobox 1)in U87 glioma cells upon glucose and glutamine deprivation in relation to inhibition of IRE1.We demonstrated that glutamine deprivation leads to up-regulation of the expression of EPAS1, TBX3, GTF2B, and MAZ genes and down-regulation of E2F8, GTF2F2, TCF8, and TBX2 genes in control glioma cells.At the same time, glucose deprivation enhances the expression of EPAS1 and GTF2B genes and decreases of E2F8, HOXC6, TCF3, and TBX2 genes in these glioma cells. Inhibition of IRE1 by dnIRE1 significantly modifies the expression most of studied genes with different magnitude. Present study demonstrates that fine-tuning of the expression of proliferation related transcription factor genes depends upon glucose and glutamine deprivation in IRE1-dependent manner and possibly contributes to slower tumor growth after inhibition of IRE1.

Key words: mRNA expression;transcription factors; endoplasmic reticulum stress;IRE1 inhibition; glucose deprivation; glutamine deprivation;glioma cells.

INTRODUCTION

Multiple studies have clarified the link between endoplasmic reticulum stress and cancer [1-4]. Malignant tumors use endoplasmic reticulum stress response and its signaling pathways to adapt and to enhance tumor cells proliferation under stressful environmental conditions [5-7]. It is well known that activation of ERN1/IRE1 (endoplasmic reticulum to nucleus signaling 1/ inositol requiring enzyme 1) branch of the endoplasmic reticulum stress response is tightly linked to apoptosis and to cell death, and suppression of its function has been demonstrated to result in significant anti-proliferative effect in glioma growth [8-11]. Glucose and glutamine are substrates for glycolysis and glutaminolysis, which are important for tumor progression through regulation of the cell cycle at distinct stages [12, 13]. The activation of glycolysis and glutaminolysis in cancer cells is tightly regulated by the action of two ubiquitin ligases, which control the transient appearance and metabolic activity of the glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) and glutaminase 1 (GLS1), the first enzyme in glutaminolysis [12].

Malignant gliomas are highly aggressive tumors and to date there is no effective treatment available. As solid tumors expand, oxygen and nutrients become limiting owing to inadequate vascularization. Glucose shortage associated with malignant progression triggers apoptosis through the endoplasmic reticulum unfolded protein response [14]. Moreover, endoplasmic reticulum stress is in part caused by reduced glucose flux [14]. Thus, a better understanding of tumor responses to glucose and glutamine deprivations as well as to endoplasmic reticulum stress is required to elaborate therapeutical strategies of cell sensibilization, based on the blockade of survival mechanisms [4, 14-18].

The endoplasmic reticulum is a key subcellular structure in the response to different factors, which activate a complex set of signaling pathways, named the unfolded protein response and plays an important role in metabolic integration [5, 6, 19]. This adaptive response is mediated by three interconnected endoplasmic reticulum-resident sensors, but IRE1 is the most evolutionary conversed and an important sensor of the unfolded protein response to the accumulation of misfolded proteins and represents a key regulator of the life and death processes [1, 6, 7, 11]. The IRE1 enzyme contains protein kinase and endoribonuclease activities. The protein kinase of IRE1 responsible for autophosphorylation and controls some gene expressions [7, 20]. The IRE1 endoribonuclease activity is involved in the degradation of a specific subset of mRNA and also initiates the cytosolic splicing of the pre-XBP1 (X-box binding protein 1) mRNA whose mature transcript encodes a transcription factor that stimulates the expression of numerous unfolded protein response specific genes [11, 21-24].

Transcription factors are responded to diverse cellular stresses to regulate expression of its target genes, thereby inducing cell cycle control, proliferation, apoptosis, and senescence, especially in cancer [25-30]. E2F family of transcription factors regulates various cellular functions related to cell cycle and apoptosis and is strongly up-regulated in human hepatocellular carcinoma, thus possibly contributing to hepatocarcinogenesis [25, 26]. Moreover, there is data that transcription factors E2F7 and E2F8 promote angiogenesis through transcriptional activation of VEGFA in cooperation with HIF1 [27]. The T-box transcription factors TBX3 and TBX2 play multiple roles in normal development and disease by either repressing or activating transcription of target genes in a context-dependent manner and control the rate of cell proliferation as well as mediate cellular signaling pathways [28]. It was shown that TBX3 is a transcriptional repressor and its overexpression is associated with several cancers, but this transcription factor may mediate the antiproliferative and promigratory role of TGF-β1 in breast epithelial and skin keratinocytes [29].

Transcription factor HOXC6 is a member of a highly conserved homeobox family of transcription factors that play an important role in proliferation as well as in morphogenesis and metastasis and regulates genes with both oncogenic and tumor suppressor activities and may contribute to the progression of gastric carcinogenesis [30]. Recently was shown that knockdown of endothelial PAS domain protein 1 (EPAS1), which also known as hypoxia-inducible transcription factor-2alpha (HIF-2α), as well as HIF-1α decreased cell proliferation under normoxic as well as hypoxic conditions in pulmonary vascular endothelial cells and that EPAS1/HIF-2α and SOX9 regulate *TUBB3* gene expression and affect ovarian cancer aggressiveness [31]. Moreover, the expression of *EPAS1* gene was significantly correlated with tumor size, invasion, and necrosis as well as with *VEGF* gene expression, which supported the correlation of EPAS1 up-regulation with tumor angiogenesis [32].

Recently was shown that genes encoded transcription factors E2F8, HOXC6, EPAS1, and TBX3 are strongly depended from the endoplasmic reticulum stress particularly its IRE1 signaling pathway, because inhibition of IRE1, especially its endoribonuclease activity,

significantly affects all these gene expressions and this deregulation of proliferation related genes (suppression of pro-proliferative genes *E2F8, HOXC6,* and *EPAS1* and up-regulation of transcription repressor gene *TBX3*) correlates to slower tumor growth [33].

The general transcription factor IIb (GTF2B) is also overexpression in human hepatocellular carcinoma and has a potential link to cell proliferation [34]. There is data that the MYC-associated zinc-finger protein MAZ is a purine-binding transcription factor, which modulates cell proliferation and metastasis through reciprocal regulation of androgen receptor [35]. The transcriptional repressor SNAI2 (snail family zinc finger 2), also known as SLUG, is controlled invasion of colorectal cancer cells [36]. Recently was shown that CUL4A induces epithelial-mesenchymal transition and promotes cancer metastasis by regulating the expression of transcriptional repressor TCF8/ZEB1 (zinc finger E-box binding homeobox 1) [37].

The aim of this study was to investigate the effects of glucose and glutamine deprivation on the expression of genes encoded transcription factors E2F8, EPAS1/HIF2A, HOXC6, TBX3, TBX2, GTF2B, GTF2F2, MAZ, SNAI2, TCF3, and TCF8/ZEB1, which participate in the regulation of cell proliferation and invasion in U87 glioma cell line and its subline with IRE1 loss of function for evaluation of possible significance these genes in the control of tumor growth through IRE1-mediated endoplasmic reticulum stress signaling.

METHODS

The glioma cell line U87 (HTB-14) was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37ºC in a 5% CO_2 incubator. Glucose and glutamine deprivation conditions were created by changing the complete DMEM medium into culture plates on DMEM medium without glucose or glutamine and plates were exposed to these conditions for 16 h.

In this work we used two sublines of U87 glioma cells. One subline was obtained by selection of stable transfected clones with overexpression of vector (pcDNA3.1), which was used for creation of dominant-negative construct (dnIRE1). This untreated subline of glioma cells was used as control (control glioma cells) in the study of the effects of glutamine and glucose deprivations on the expression level of transcription factor *E2F8, EPAS1, HOXC6,TBX3, TBX2, GTF2F2, GTF2B, MAZ, SNAI2,TCF3,* and *TCF8/ZEB1* genes. Second subline was obtained by selection of stable transfected clones with overexpression of dnIRE1 and has suppressed both protein kinase and endoribonuclease activities of this bifunctional signaling enzyme of endoplasmic reticulum stress. This cell subline is a gift from prof. M. Moenner (France) [4]. Previously was shown that these cells have low proliferation rate and does not express XBP1 alternative splice variant, a key transcription factor in IRE1 signaling, after induction endoplasmic reticulum stress by tunicamycin [6].The expression level of the studied genes in these cells upon glutamine and glucose deprivations was compared with cells, transfected by vector (control glioma cells).

Total RNA was extracted from glioma cells using Trizol reagent according to manufacturer protocols (Invitrogen, USA). The RNA pellets were washed with 75 % ethanol and dissolved in nuclease-free water. For additional purification RNA samples were re-precipitated with 95 % ethanol and re-dissolved again in nuclease-free water.

QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to manufacturer protocol. The expression level of *E2F8, EPAS1, HOXC6, TBX3, TBX2, GTF2F2, GTF2B, MAZ,*

SNAI2, TCF3, TCF8, and ACTB mRNA were measured in U87 glioma cells by real-time quantitative polymerase chain reaction (qPCR) using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific, ABgene House, UK) or semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) using"MasterCycler Personal" ("Eppendorf", Germany). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich (USA).

For amplification of HOXC6 (homeobox C6 transcription factor) cDNA we used next forward and reverse primers: 5'– AAAAG AGGAAAAGCGGGAAG–3' and (5'– GGTCC ACGTTTGACTCCCTA –3', correspondingly. The nucleotide sequences of these primers correspond to sequences 772 – 791 and 963 – 944 of human HOXC6 cDNA (GenBank accession number NM_004503). The size of amplified fragment is 192 bp. The amplification of EPAS1 (endothelial PAS domain protein 1), also known as hypoxia-inducible factor 2alpha (HIF-2α), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– AAGCCTTGGAGGGTTTCATT–3' and reverse – 5'– TCATGAAGAAGTCCCGCTCT –3'. The nucleotide sequences of these primers correspond to sequences 788 – 807 and 1021 – 1002 of human EPAS1 cDNA (GenBank accession number NM_001430). The size of amplified fragment is 234 bp. For amplification of E2F8 (E2F transcription factor 8)cDNA we used next forward and reverse primers: 5'– CCACCACAGCAAATATCGTF–3' and 5'– CTTTGGCCTCAGGTAATCCA –3', correspondingly. The nucleotide sequences of these primers correspond to sequences 596 – 615 and 805 – 786 of human E2F8 cDNA (GenBank accession number NM_024680). The size of amplified fragment is 210 bp. For amplification of TBX3 (T-box 3 transcriptional repressor) cDNA we used forward (5'– ACTGGG GAACAGTFGATGTC–3' and reverse (5'– TTCGGGGAACA AGTATGTCC–3') primers. The nucleotide sequences of these primers correspond to sequences 1551 – 1570 and 1729 – 1710 of human TBX3 cDNA (GenBank accession number NM_005996). The size of amplified fragment is 179 bp. For amplification of TBX2 (T-box 2 transcriptional factor) cDNA we used forward (5'– GGGAC CAGTTCCACAAGCTA–3' and reverse (5'– AAGCCGTFCTTGTCAGAGAT–3') primers. The nucleotide sequences of these primers correspond to sequences 625 – 644 and 943 – 924 of human TBX2 cDNA (GenBank accession number NM_005994). The size of amplified fragment is 319 bp. The amplification of GTF2F2 (general transcription factor IIF, polypeptide 2), also known as ATP-dependent helicase GTF2F2 and transcription initiation factor RAP30, cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– GGAGTFTGGCTAGTCAAGGT–3' and reverse – 5'– GCACTGACTGAAGCTG GTTT–3'. The nucleotide sequences of these primers correspond to sequences 209 – 228 and 390 – 371 of human GTF2F2 cDNA (GenBank accession number NM_004128). The size of amplified fragment is 182 bp. For amplification of GTF2B (general transcription factor IIB), also known as RNA polymerase II transcription factor IIB, cDNA we used forward (5'– TCTGTTG TFTCTTGTTGCGG–3' and reverse (5'– GTTCGCCATTCAGATCCCAC–3') primers. The nucleotide sequences of these primers correspond to sequences 80 – 99 and 280 – 261 of human GTF2B cDNA (GenBank accession number NM_001514). The size of amplified fragment is 201 bp. The amplification of MAZ (MYC-associated zinc finger protein, purine-binding transcription factor), also known as serum amyloid A activating factor 1 (SAF-1), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– TCTACCACCTGAACCGAC AC –3' and reverse – 5'– TTTGAAGGGC CGTTCTGTTG–3'. The nucleotide sequences of these primers correspond to sequences 1037 – 1056 and 1266 – 1247 of human

MAZ cDNA (GenBank accession number NM_002383). The size of amplified fragment is 230 bp. For amplification of transcription factor SNAI2 (snail family zinc finger 2), also known as neural crest transcription factor SLUG, cDNA we used forward (5'– CCT GGTTGCTTCAAGGACAC–3' and reverse (5'–AGCAGCCAGATTCCTCATGT–3') primers. The nucleotide sequences of these primers correspond to sequences 765 – 784 and 968 – 949 of human SNAI2 cDNA (GenBank accession number NM_003068). The size of amplified fragment is 204 bp. The amplification of transcriptional regulator TCF3 (transcription factor 3), also known as E2A immunoglobulin enhancer binding factors E12E47, cDNA was performed using forward primer (5'– ACAAGGAGCTCAGTFACCTC–3') and reverse primer (5'– CTGTFCGACTCAGTFA AGTF–3'). These oligonucleotides correspond to sequences 107 – 126 and 326 – 307 of human TCF3 cDNA (GenBank accession number NM_003200).The size of amplified fragment is 220 bp. The amplification of transcription repressor TCF8 (factor 8), also known as ZEB1 (zinc finger E-box binding homeobox 1), cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– CAGGGAGGAGCAGTFAAAGA –3' and reverse – 5'– CTCTTCAGGTFCCTCAGGAA –3'.The nucleotide sequences of these primers correspond to sequences 209 – 228 and 438 – 419 of human TCF8/ZEB1 cDNA (GenBank accession number NM_030751). The size of amplified fragment is 230 bp. The amplification of beta-actin (ACTB) cDNA was performed using forward - 5'– GGACTTC GAGCAAGAGATGG –3' and reverse - 5'– AGCACTGTFTTGGCGTACAG –3' primers. These primer nucleotide sequences correspond to 747 – 766 and 980 – 961 of human *ACTB* cDNA (GenBank accession number NM_001101). The size of amplified fragment is 234 bp. The expression of beta-actin mRNA was used as control of analyzed RNA quantity.

An analysis of quantitative PCR was performed using special computer program Differential Expression Calculator. The values of the expression of transcription factors E2F8, EPAS1, HOXC6, TBX3, TBX2, GTF2F2, GTF2B, MAZ, SNAI2, TCF3, and TCF8 mRNA were normalized to beta-actin mRNA expressions and represent as percent of control (100 %). Statistical analysis was performed according to Student's test using Excel program and OriginPro 7.5 software. All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments.

RESULTS

To test the effect of glutamine and glucose deprivations on expression levels of different transcription factor genes, strongly related to both positive and negative control of cell proliferation in relation to IRE1 signaling enzyme function, we used the U87 glioma cell sublines, which constitutively expresses vector pcDNA3.1 (control cells) or dnIRE1 [10, 21]. Figure 1 demonstrates that glutamine deprivation affects the expression of studied genes at mRNA level: up-regulates the expression of *EPAS1* (+28 %), *TBX3* (+26 %), *MAZ* (+22 %), and *GTF2B* (+51 %) genes and down-regulates of *E2F8* (-50 %), *TBX2* (-47 %), *TCF8/ZEB1* (-16 %), and *GTF2F2 (*-27 %) gene expressions as compared to control glioma cells growing with glutamine. It is interesting to note that more significant changes in the expression level were shown for *E2F8, TBX2,* and *GTF2B* genes as compared to other studied genes. At the same time, the expression of *HOXC6, SNAI2,* and *TCF3* genes at mRNA level was resistant to glutamine deprivation in control glioma cells.

We also analyzed the expression level of genes encoded different transcription factors in glioma cells upon glucose deprivation condition. As shown in Figure 2, glucose deprivation up-regulates the expression level of *EPAS1* (+45 %) and *GTF2B* (+16 %) genes only and down-regulates of *E2F8* (2.9 fold), *TBX2* (-34 %), *TCF3* (-22 %), and *HOXC6* (-15 %) gene expressions in control

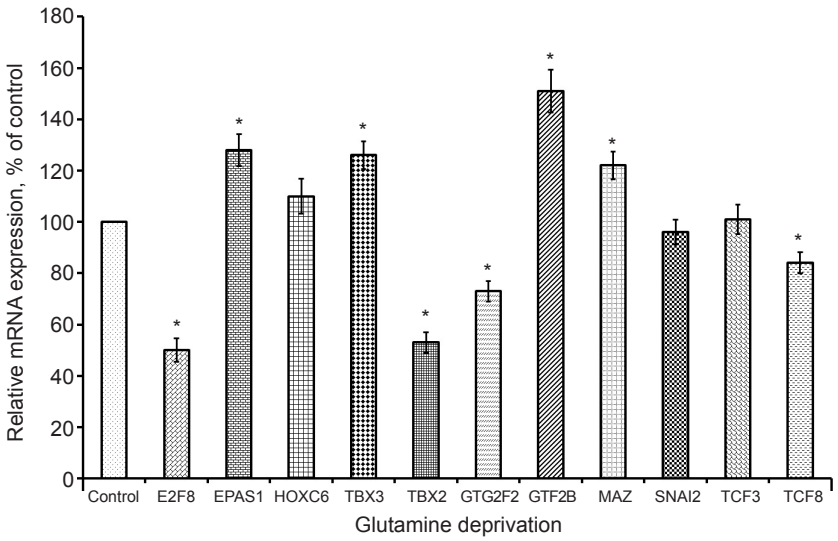
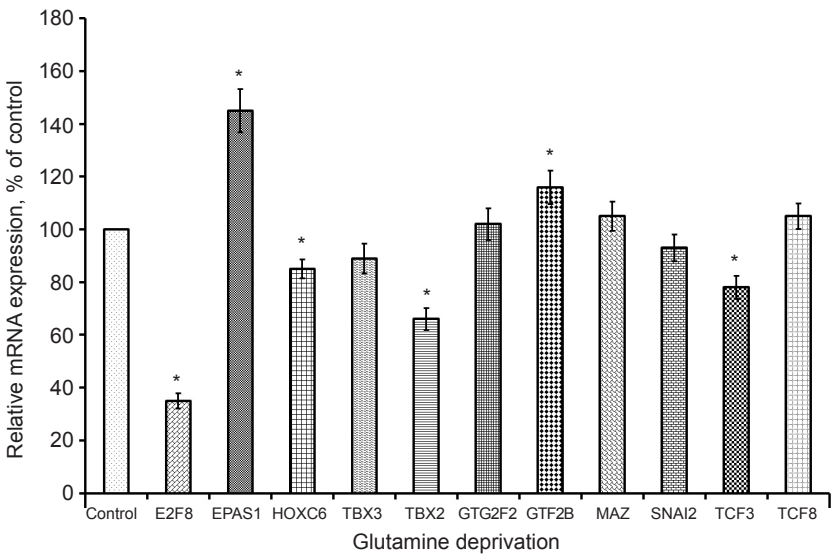


Figure 1. Effect of glutamine deprivation on the expression of transcription factor genes *E2F8* (E2F transcription factor 8)*, EPAS1* (endothelial PAS domain protein 1)*, HOXC6* (homeobox C6), *TBX3* (T-box 3), *TBX2* (T-box 2), *GTF2F2* (general transcription factor IIF), *GTF2B* (general transcription factor IIB), *MAZ* (MYC-associated zinc finger protein, purine-binding transcription factor), *SNAI2* (snail family zinc finger 2), *TCF3* (transcription factor 3), and *TCF8* in glioma cell line U87 measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of control (vector, 100 %); mean ± SEM; ***n*** = 4; * – $P < 0.05$ as compared to control

glioma cells as compared to cells growing with glucose. It is interesting to note that more significant changes in the expression level were shown for *E2F8* and *EPAS1* genes as compared to other studied genes. At the same time, the expression of *GTF2F2, TBX3, MAZ, SNAI2,* and *TCF8* genes at mRNA level was resistant to glucose deprivation in control glioma cells.

We next studied how inhibition of IRE1 modulates the effect of glutamine and glucose

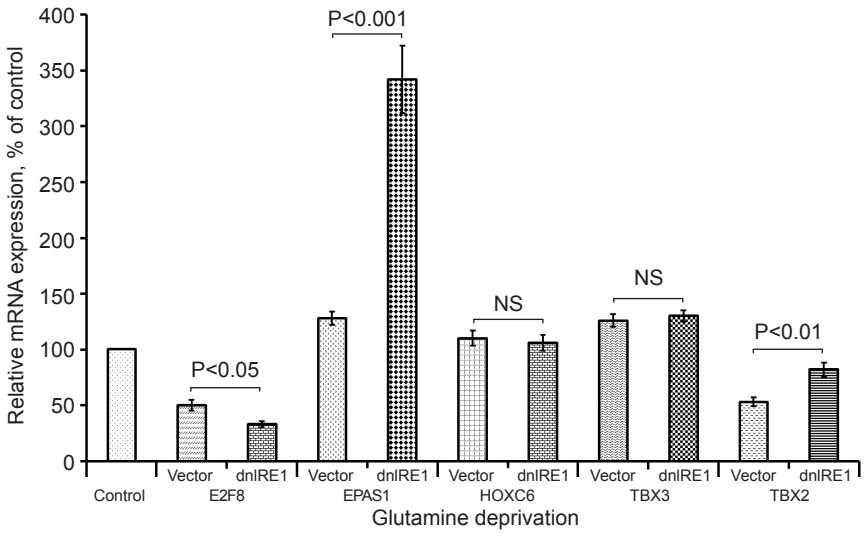


Figures 2. Effect of glucose deprivation on the expression of transcription factor genes *E2F8, EPAS1, HOXC6*, *TBX3*, *TBX2*, *GTF2F2*, *GTF2B*, *MAZ*, *SNAI2*, *TCF3*, and *TCF8* in glioma cell line U87 measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of control (vector, 100 %); mean ± SEM; ***n*** = 4; * – $P < 0.05$ as compared to control

deprivation on the expression of these genes on mRNA level. As shown in Figure 3, the expression of *HOXC6* gene is resistant to glutamine deprivation condition in both control glioma cells and cells with knockdown IRE1. However, the changes in the expression of genes encoded transcription factor EPAS1, E2F8, and TBX2 upon glutamine deprivation condition is significantly different in control glioma and cells with knockdown IRE1: for EPAS1 in 8.6 fold, for E2F8 in 1.3 fold, and for TBX2 in 2.6 fold. Moreover, inhibition of IRE1 completely abolishes the effect of glutamine deprivation on the expression *TBX2* gene.

The results of comparative investigation of the expression of *GTF2F2, GTF2B, MAZ, SNAI2,TCF3,* and *TCF8/ZEB1* genes upon glutamine deprivation in two types of glioma cells are shown in Figure 4. Thus, the expression of *TCF3* gene is resistant to glutamine deprivation condition in both control glioma cells and cells with knockdown IRE1. At the same time, glutamine deprivation down-regulates the expression level of *GTF2F2* gene in both control glioma cells and in cells without IRE1 enzyme function, but these changes are similar (Figure 4). Moreover, inhibition of IRE1 creates sensitivity of *SNAI2* gene to glutamine deprivation and abolishes the effect of this deprivation on the expression of MAZ gene as well as enhances the effect of glutamine deprivation on the expression of *GTF2B* (1.5 fold) and *TCF8/ZEB1* (1.9 fold) genes.

We also studied the effect of inhibition of IRE1 signaling enzyme on sensitivity of the expression of transcription factor genes at mRNA level to glucose deprivation in glioma cells. As shown in Figure 5, the expression of *TBX3* gene is resistant to glucose deprivation condition in both control glioma cells and cells with knockdown IRE1. At the same time, glucose deprivation down-regulates the expression level of *TBX2* gene only in control glioma cells and inhibition of IRE1 enzyme function abolishes the sensitivity of this gene expression to glucose deprivation (Figure 5). Moreover, the changes in the expression level of genes encoded transcription factor *E2F8, EPAS1,* and *HOXC6* upon glucose deprivation condition is significantly different in control glioma and cells with knockdown IRE1: for *E2F8* in 2.1 fold, for *EPAS1* in 5.9 fold, and for *HOXC6* in 2.5 fold.



Figures 3. Comparative effect of glutamine deprivation on the expression of transcription factor genes *E2F8, EPAS1, HOXC6*, *TBX3*, and *TBX2* in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of corresponding control (both controls is accepted as 100 %); NS – no significant changes; mean ± SEM; ***n*** = 4

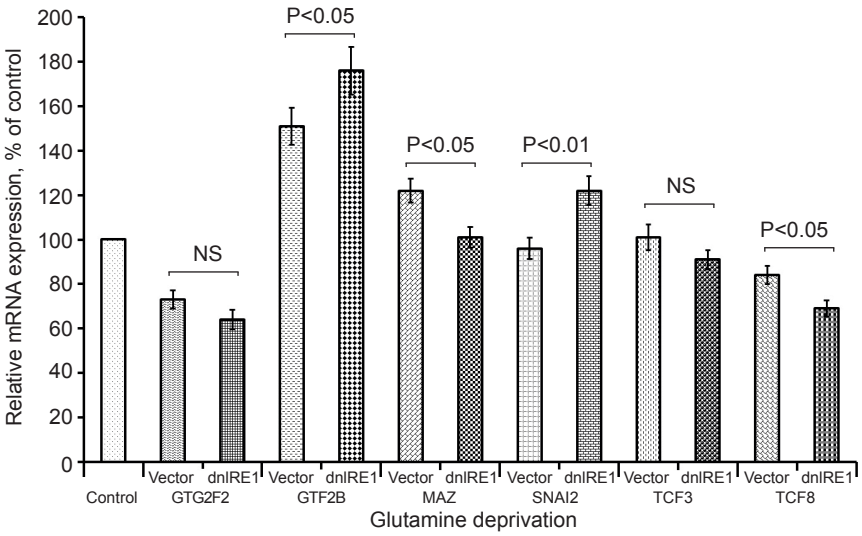
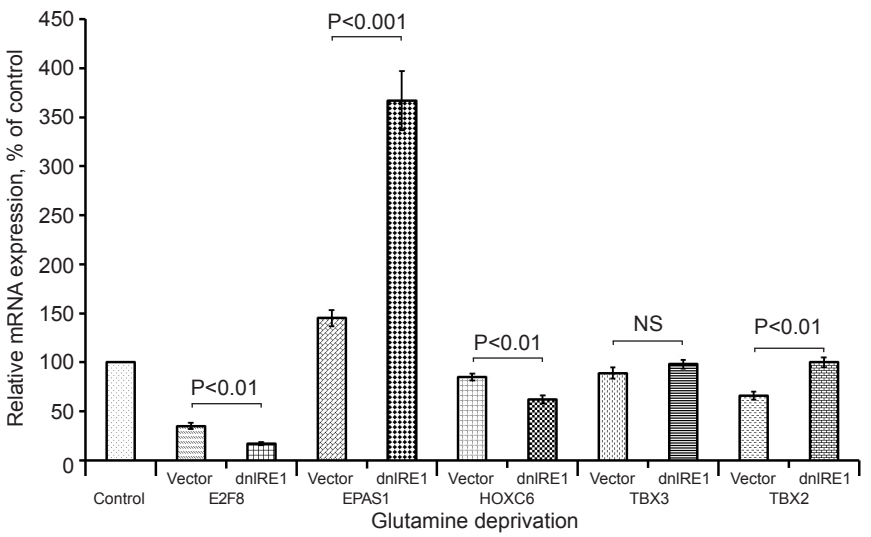


Figure 4. Comparative effect of glutamine deprivation on the expression of transcription factor genes *GTF2F2*, *GTF2B*, *MAZ*, *SNAI2*, *TCF3*, and *TCF8* in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of corresponding control (both controls is accepted as 100 %); NS – no significant changes; mean ± SEM; ***n*** = 4

Figure 6 contains the results of comparative study the sensitivity of *GTF2F2, GTF2B, MAZ, SNAI2,TCF3,* and *TCF8/ZEB1* gene expressions to glucose deprivation in control glioma cells and cells without IRE1 signaling enzyme function. We have shown that the expression of *TCF8* gene is resistant to glucose deprivation condition in both control glioma cells and cells with IRE1 knockdown. At the same time, the expression of MAZ gene is also resistant to glucose deprivation in both types of cells; however, between these two groups there is



Figures 5. Comparative effect of glucose deprivation on the expression of transcription factor genes *E2F8, EPAS1,HOXC6*, *TBX3*, and *TBX2* in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of corresponding control (both controls is accepted as 100 %); NS – no significant changes; mean ± SEM; ***n*** = 4

small but statistically significant differences (Figure 6). We have also shown that inhibition of IRE1 creates sensitivity of *GTF2F2* and *SNAI2* genes to glucose deprivation and enhances the effect of this deprivation on the expression of *GTF2B* gene (4.4 fold). Furthermore, inhibition of IRE1 signaling enzyme does not change the effect of glucose deprivation on the expression *TCF3* gene: -22 % in control glioma cells and -20 % in IRE1 knockdown cells (Figure 6).

Thus, the expression almost all studied genes encoded proliferation-related transcription factors is affected by glutamine and glucose deprivations and inhibition of IRE1 preferentially modified sensitivity of these gene expressions to glutamine and glucose deprivations.

DISCUSSION

Results of this study clearly demonstrated that the expression levels of almost all tested genes (*E2F8, EPAS1, HOXC6,TBX3, TBX2, GTF2F2, GTF2B, MAZ, SNAI2,TCF3,* and *TCF8/ZEB1*) encoding key proliferation-related transcription factors, which are stress responsible and participate in malignant tumor growth, are affected by glucose and glutamine deprivations through IRE1 signaling branch of endoplasmic reticulum stress. Our results are confirmed to data that glycolysis and glutaminolysis are related to the control of cell proliferation through regulation of cell cycle and tumor suppressor genes [12, 38-40]. Recently we have shown that genes encoded transcription factorsE2F8, HOXC6, EPAS1, and TBX3are strongly depended from the endoplasmic reticulum stress particularly its IRE1 signaling pathway, because inhibition of IRE1, especially its endoribonuclease activity, significantly affects all these gene expressions. Furthermore, this deregulation of proliferation related transcription factorgenes (suppression of pro-proliferative genes *E2F8, HOXC6,* and *EPAS1* and up-regulation of transcription repressor gene *TBX3*) strongly correlates to down-regulation of glioma cell proliferation and slower tumor growth [33].

In this study we have shown that both glucose and glutamine deprivation gown-regulates the expression level of *E2F8* gene and that inhibition of IRE1 signaling enzyme function in U87 glioma cells more strongly decreases

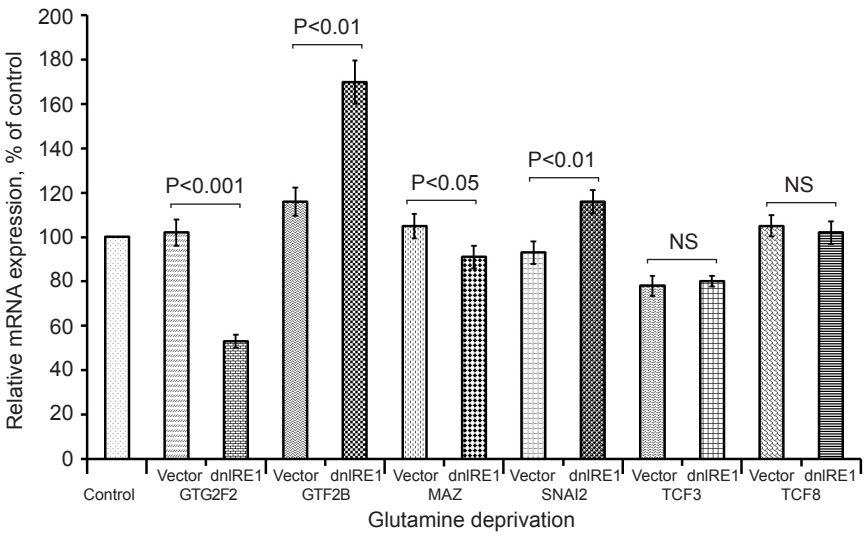


Figure 6. Comparative effect of glucose deprivation on the expression of transcription factor genes *GTF2F2*, *GTF2B*, *MAZ*, *SNAI2*, *TCF3*, and *TCF8* in two types of glioma cells: control cells transfected by vector (Vector) and cells with a deficiency of the signaling enzyme IRE1 (dnIRE1) measured by quantitative PCR. Values of these gene expressions were normalized to beta-actin expression and represent as percent of corresponding control (both controls is accepted as 100 %); NS – no significant changes; mean ± SEM; ***n*** = 4

E2F8 gene expression and observed down-regulation correlate with suppression of this gene expression and cell proliferation by IRE1 inhibition [33]. Thus, our results completely argue to data Colombo et al. [12], which shown that glucose and glutamine are required for tumor progression through cell cycle control and that deprivation of these substrates of glycolysis and glutaminolysis have opposite effect. There is also data that E2F8 creates complexes with other members of E2F family of transcription factors and coordinates various cellular functions through the regulation of the expression of target genes, thereby regulating cell cycle, apoptosis, and angiogenesis, including transcriptional activation of VEGFA in cooperation with HIF1 [25-27].

At the same time, both glutamine and glucose deprivations lead to up-regulation of the expression level of transcription factor EPAS1/HIF-2α and inhibition of IRE1 enhances effect of glutamine and glucose deprivation conditions on this gene expression. The expression of *EPAS1* gene mediates numerous hypoxia-induced processes including proliferation in cell-specific manner is significantly correlated with tumor size, invasion, and necrosis as well as with *VEGF* gene expression, which supported the correlation of EPAS1 up-regulation with tumor angiogenesis [31, 32]. Moreover, *EPAS1* gene expression level is strongly down-regulated in glioma cells harboring dnIRE1 [33]. Thus, biological significance of increased expression of *EPAS1* gene upon glutamine and glucose deprivation is not clear yet and warrants further study.

We have also demonstrated that the expression of T-box transcription factor TBX3 is elevated in glioma cells exposure to glutamine deprivation condition, but is resistant to glucose deprivation. Moreover, up-regulation of *TBX3* gene expression was observed in glioma cells growing in regular medium (with glucose and glutamine) when IRE1 function is inhibited [33\. This increase of *TBX3* gene expression by IRE1 inhibition as well as by glutamine deprivation may contribute to the suppression of cell proliferation and glioma growth of these cells, because TBX3 is a transcriptional repressor, which controls the rate of cell proliferation as well as mediate cellular signaling pathways and high expression level of this gene was found in normal human astrocytes [28, 30, 33]. At the same time, transcription factor TBX3 plays multiple roles in normal development and disease by either repressing or activating transcription of target genes in a context-dependent manner and it may mediate the antiproliferative and pro-migratory role of TGF-β1 in breast epithelial and skin keratinocytes, but its overexpression is associated with several cancers [29]. Thus, increased expression of TBX3 can mediate inhibition of cell growth and could also contribute to the suppression of glioma cells proliferation, since it has pleiotropic functions. Transcription factor TBX2 has preferentially opposite functions to TBX3 and its expression is down-regulated upon both glutamine and glucose deprivation possibly through IRE1 signaling pathway, because inhibition of IRE1 abolishes sensitivity of this gene expression to glutamine as well as glucose deprivation condition. Thus, decreased expression of transcription factor TBX2 can mediate inhibition of cell growth upon glutamine and glucose deprivation.

CONCLUSIONS

Results of this study demonstrated that the expression of almost all studied genes encoded key proliferation-related transcription factors is affected by glutamine and glucose deprivation conditions in gene-specific manner and that inhibition of IRE1, a central mediator of endoplasmic reticulum stress response, preferentially modified sensitivity of these gene expressions to glutamine and glucose deprivations and possibly contributes to slower glioma growth. However, molecular mechanisms of the regulation of proliferation-related transcription factor genes by glutamine and glucose deprivation through the endoplas-

mic reticulum stress response pathways warrant further investigation for clarification the role of glycolysis and glutaminolysis in cancer progression as well as development new strategies of anti-tumor therapy.

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ЕКСПРЕСІЯ ГЕНІВ ТРАНСКРИПЦІЙНИХ ФАКТОРІВ, ЩО МАЮТЬ ВІДНОШЕННЯ ДО ПРОЛІФЕРАЦІЇ, У КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87 З ПРИГНІЧЕНИМ IRE1 ЗА УМОВ ДЕФІЦИТУ ГЛЮКОЗИ ТА ГЛУТАМІНУ

Глюкоза та глутамін, як і стрес ендоплазматичного ретикулума, є необхідними для росту пухлин, оскільки причетні до регуляції клітинного циклу. Пригнічення ERN1/IRE1 (сигналювання від ендоплазматичного ретикулума до ядра 1/залежний від інозитолу ензим 1), що є центральним медіатором стресу ендоплазматичного ретикулума, істотно пригнічує проліферацію клітин гліоми і ріст пухлин, а також модифікує чутливість експресії генів до дефіциту глюкози та глутаміну. Ми вивчали експресію генів, що кодують такі транскрипційні фактори, як E2F8 (E2F transcription factor 8), EPAS1 (endothelial PAS domain protein 1), HOXC6 (homeobox C6), TBX3 (T-box 3), TBX2 (T-box 2), GTF2F2 (general transcription factor IIF), GTF2B (general transcription factor IIB), MAZ (MYC-associated zinc finger protein, purine-binding transcription factor), SNAI2 (snail family zinc finger 2), TCF3 (transcription factor 3) та TCF8/ZEB1 (zinc finger E-box binding homeobox 1) у клітинах гліоми лінії U87 за умов дефіциту глюкози та глутаміну залежно від пригнічення IRE1. Нами встановлено, що за умов дефіциту глутаміну спостерігається посилення експресії генів *EPAS1, TBX3, GTF2B* та *MAZ*і зниження експресії генів *E2F8, GTF2F2, TCF8* та *TBX2* у контрольних клітинах гліоми. Водночас за умов дефіциту глюкози збільшується рівень експресії генів *EPAS1* та *GTF2B* і зменшується - *E2F8, HOXC6, TCF3* та *TBX2* у цих клітинах гліоми. Пригнічення IRE1 за допомогою dnIRE1 суттєво змінює експресію більшості досліджених генів, але по-різному. Ця робота продемонструвала, що експресія генів транскрипційних факторів, що мають відношення до проліферації, змінюються за умов дефіциту глюкози та глутаміну залежно від функції IRE1 і можливо задіяні у зниженні інтенсивності росту пухлин після пригнічення IRE1.

Ключові слова: експресія мРНК; транскрипційні фактори; стрес ендоплазматичного ретикулума; пригнічення IRE1; дефіцит глюкози; дефіцит глутаміну; клітини гліоми.

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ЭКСПРЕССИЯ ГЕНОВ ТРАНСКРИПЦИОННЫХ ФАКТОРОВ, КОТОРЫЕ ИМЕЮТ ОТНОШЕНИЕ К ПРОЛИФЕРАЦИИ, В КЛЕТКАХ ГЛИОМЫ ЛИНИИ U87 С УГНЕТЕННЫМ IRE1 ПРИ ДЕФИЦИТЕ ГЛЮКОЗЫ И ГЛУТАМИНА

Глюкоза и глутамин, как и стресс эндоплазматического ретикулума – необходимые факторы роста опухолей, поскольку они причастны к регуляции клеточного цикла. Угнетение ERN1/IRE1 (сигналинг от эндоплазматического ретикулума к ядру 1/зависимый от инозитола энзим 1), что является центральным медиаторомстресса эндоплазматического ретикулума, существенно угнетает пролиферацию клеток глиомы и рост опухоли, а также модифицирует чувствительность экспрессии генов к дефициту глюкозы и глутамина. Мы изучали экспрессию генов, что кодируют транскрипционные факторы E2F8 (E2F transcription factor 8), EPAS1 (endothelial PAS domain protein 1), HOXC6 (homeoboxC6), TBX3 (T-box 3), TBX2 (T-box 2), GTF2F2 (general transcription factor IIF), GTF2B (general transcription factor IIB), MAZ(MYC-associated zinc finger protein, purine-binding transcription factor), SNAI2 (snail family zinc finger 2), TCF3 (transcription factor 3) и TCF8/ZEB1 (zinc finger E-box binding homeobox 1) в клетках глиомы линии U87 при дефиците глюкозы и глутамина в зависимости от угнетения IRE1. Нами установлено, что при дефиците глутамина отмечается усиление экспрессии генов *EPAS1, TBX3, GTF2B* и *MAZ* и снижение – *E2F8, GTF2F2, TCF8* и *TBX2* в контрольных клетках глиомы. В то же время, при дефиците глюкозы отмечается увеличение уровня экспрессии генов *EPAS1* и *GTF2B* и снижение – *E2F8, HOXC6, TCF3* и *TBX2* в этих клетках глиомы. Угнетение IRE1 с помощью dnIRE1 существенно изменяет экспрессию большинства исследованных генов, но поразному. Эта работа продемонстрировала, что экспрессия генов транскрипционных факторов, что имеют отношение к пролиферации, изменяется при дефиците глюкозы и глутамина в зависимости от функции IRE1 и возможно задействованы в снижении интенсивности роста опухолей после угнетения IRE1.

Ключевые слова: экспрессия мРНК; транскрипционные факторы; стресс эндоплазматического ретикулума; угнетение IRE1; дефицит глюкозы; дефицит глутамина; клетки глиомы.

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