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IRE1 inhibition affects the expression of insulin-like growth factor binding protein genes and modifies its sensitivity to glucose deprivation in U87 glioma cells

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Objective. The aim of the present study was to investigate the effect of inhibition of endoplasmic reticulum stress signaling mediated by IRE1/ERN1 (inositol-requiring enzyme 1/endoplasmic reticulum to nucleus signaling 1) on the expression of genes encoding different groups of insulin-like growth binding proteins (*IGFBP6* and *IGFBP7*) and CCN family (*IGFBP8/CTGF/CCN2*, *IGFBP9/NOV/CCN3*, *IGFBP10/CYR61/CCN1*, *WISP1/CCN4*, and *WISP2/CCN5*) and its sensitivity to glucose deprivation in U87 glioma cells.

Methods. The expression of *IGFBP6*, *IGFBP7*, *IGFBP8*, *IGFBP9*, *IGFBP10*, *WISP1*, and *WISP2* genes was studied by qPCR in control U87 glioma cells (wild-type) and its subline with IRE1 signaling enzyme loss of function upon glucose deprivation.

Results. The expression of *IGFBP8*, *IGFBP9,* and *WISP2* genes was up-regulated in control glioma cells upon glucose deprivation with most significant changes for *IGFBP9* gene. At the same time, the expression of *IGFBP6*, *IGFBP10*, and *WISP1* genes was resistant to glucose deprivation in these glioma cells, but the *IGFBP7* gene expression was down-regulated. The inhibition of both enzymatic activities (kinase and endoribonuclease) of IRE1 in glioma cells modified the sensitivity of most studied gene expressions to glucose deprivation condition: introduced sensitivity of *IGFBP10* and *WISP1* genes to glucose deprivation, enhanced the effect of this deprivation on *IGFBP7 and IGFBP9* gene expressions, and reduced this effect on *WISP2* gene and induced suppressive effect of glucose deprivation on the expression of *IGFBP8* gene. Furthermore, the inhibition of IRE1 strongly affected the expression of all studied genes in glioma cells upon regular growing condition in gene specific manner: up-regulated the expression levels of *IGFBP7*, *IGFBP8*, *IGFBP10*, *WISP1*, and *WISP2* genes and down-regulated the *IGFBP6 and IGFBP9* genes.

Conclusions. The data of this investigation demonstrate that the expression of *IGFBP7*, *IGFBP8*, *IGFBP9*, and *WISP2* genes are sensitive to glucose deprivation in U87 glioma cells and that inhibition of IRE1 signaling enzyme function may significantly affect the expression of all studied genes in the presence of glucose as well as modify the effect of glucose deprivation on the expression of most studied genes. These data also show that proteins encoded by these genes may participate in the regulation of metabolic and proliferative processes via IGF/INS receptors and possibly other signaling pathways as well, via IRE1 signaling, which is a central mediator of the unfolded protein response and an important component of the tumor growth and metabolic diseases.

Key words: IGFBP6, IGFBP7, IGFBP8, IGFBP9, IGFBP10, WISP1, WISP2, mRNA expression, IRE1 inhibition, U87 glioma cells, glucose deprivation

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Insulin-like growth factor binding proteins (IGFBPs) play an important role in the regulation of numerous metabolic and proliferative processes through interaction with the insulin-like growth factors (IGFs, IGF1, and IGF2), their cell surface receptors. Insulin receptors alter the half-life of the IGFs by modifying their biological activity. IGFs and the signal transduction networks play important role in tumorigenesis and metastasis as well as obesity and diabetes (Chitnis et al. 2008; Pollak 2008). IGFBPs participate in endoplasmic reticulum (ER) stress, which is an important factor of tumor growth, insulin resistance, and obesity (Ozcan et al. 2004; Cao and Kaufman 2013; Kuijjer et al. 2013; Yuzefovych et al. 2013; Han and Kaufman 2014; Lee and Ozcan 2014; Minchenko et al. 2015a). It is interesting to note that there exists a cross talk between IGF and insulin (INS) receptor signaling pathways at the receptor level or downstream signaling level (Limesand et al. 2013). This cross talk significantly influenced a variety of cancers because of insulin receptor isoforms. Formation of hybrid receptor isoforms between receptors for IGF1 and insulin, which are sensitive to the stimulation of all three axis ligands, as well as hybrid receptors of IGF1/insulin receptor with other tyrosine kinase potentiate the transformation of cells, tumorigenesis, and tumor neovascularization (Weroha and Halushka 2012; Grkovic et al. 2013; Kuijjer et al. 2013; Baxter 2014; Singh et al. 2014; Zhu et al. 2014; Chen et al. 2015).

The IGFBPs including CCN family proteins have IGF binding domains but different affinity to IGFs. They bind and regulate the availability of both IGFs and inhibit or stimulate the growth promoting effects of the IGFs through IGF/INS receptors and through other signaling pathways and regulate cell proliferation and survival as well as angiogenesis and cancer cell migration. Moreover, both negative and positive correlations between levels of IGF-1/IGF-1-R and clinical outcomes in head and neck cancer have been reported (Limesand et al. 2013). IGFBPs are now understood to have many actions beyond their endocrine role in IGF transport (Azar et al. 2011; Foulstone et al. 2013; Baxter 2014; Zhu et al. 2014). IGFBPs also function in the cells and extracellular matrix to regulate cell proliferation and survival and are involved in tumor development, progression, and resistance to treatment. Because they interact with many proteins, in addition to their canonical ligands (IGF1 and IGF2), they play an important role in the regulation of various processes including transcription (Ingermann et al. 2010; Baxter 2014; Ellis et al 2014; Tomblin et al 2014). Thus, IGFBP6 preferentially binds to IGF2 and has IGF-independent effects, including inhibition of angiogenesis and promotion of cancer cell migration (Bach et al. 2013). Moreover, IGFBP6 regulates cell proliferation and apoptosis: down-regulation of this gene expression leads to inhibition of cell proliferation and increased apoptotic cell death (Micutkova et al. 2011). It is interesting to note that apoptosis can be also regulated through IGF1 receptor (Pan et al. 2014). However, there are data indicating that IGFBP6 can also suppress the cellular proliferation (Raykha et al. 2013).

IGFBP7, which is also known as an insulin-like growth factor binding protein-related protein 1 (IGFBPRP1), belongs to the IGFBP family whose members have a conserved structural homology. It has a low affinity for IGFs and high affinity for insulin, suggesting that IGFBP7 may have a biological function distinct from other members of the IGFBP family. IGFBP7 has diverse biological functions, regulating cell proliferation, apoptosis, and senescence. It may also play a key role in vascular biology (Zhu et al. 2014). It elicits its biological effects by both insulin/IGF-dependent as well as -independent mechanisms. It has been also shown that IGFBP7 can bind to unoccupied IGF-1-R, block its activation by IGFs and suppress downstream signaling, thereby inhibiting protein synthesis, cell growth, and survival (Evdokimova et al. 2012; Verhagen et al. 2014). Increasing evidence exists that IGFBP7 may act as a tumor suppressor (Zhu et al. 2014; Gambaro et al. 2015). Furthermore, restoration of IGFBP7 increases radiosensitivity and chemosensitivity in hormone-refractory human prostate cancer (PC-3 cells) (Seki et al. 2013).

The CCN family of proteins includes six members originally designated CYR61, CTGF, NOV, WISP-1, WISP-2, and WISP-3 exhibiting different biological functions. They are involved in both normal and pathological processes (Perbal 2013). Moreover, these regulatory proteins are associated with the extracellular matrix and play an important role in the cancer development. These small-secreted cysteine-rich proteins contain the IGF-binding domain and mediate diverse developmental processes relevant to malignant transformation. Among them, IGFBP8/CTGF/CCN2 (connective tissue growth factor), IGFBP9/NOV/CCN3 (nephroblastoma overexpressed), and IGFBP10/CYR61/CCN1(cysteine-rich angiogenic inducer 61) play a role in cell growth regulation and are involved in angiogenesis, inflammation, matrix remodeling, proliferation, and migration/invasion, which are triggered by different signaling pathways (Gressner et al. 2011; Jim Leu et al. 2013; Wagener

et al. 2013; Dobson et al. 2014; Cheng et al. 2015; Liu et al. 2015a). Thus, CTGF is identified as a hepatocellular negative acute phase protein, which is down-regulated by IL-6 via the STAT3 pathway through interaction on the DNA binding level (Gressner et al. 2011). Furthermore, CTGF can bind vascular endothelial growth factor (VEGF) and inhibit VEGF-induced angiogenesis (Inoki et al. 2002). Recently, Dobson et al. (2014) have shown that IGFBP9/NOV/CCN3, which protects cells from invasion, is a target of hsa-mir-30c and mediated by this miRNA inhibition of NOV levels promotes the invasive phenotype of metastatic breast cancer cells. Moreover, this protein is a transcriptional target of FoxO1, a prominent mediator of insulin signaling in pancreatic β-cells, impairs pancreatic β-cell proliferation, glucose oxidation, and insulin secretion (Paradis et al. 2013). There are data indicating that IGFBP10/CYR61/CCN1 may promote growth of glioblastoma (Cheng et al. 2015) as well as suppress hepatocarcinogenesis by inhibiting compensatory proliferation (Chen et al. 2015). Furthermore, this protein is responsible for induction of the tumor epithelial-mesenchymal transition, invasion, and metastasis (Hou et al. 2014; Liu et al. 2015b).

The WNT1 (wingless-type MMTV integration site family, member 1)-inducible signaling pathway (WISP) proteins WISP1/CCN4 and WISP2/CCN5 are downstream in the WNT1 signaling pathway and are relevant to malignant transformation, cancer cell invasion, and motility (Haque et al. 2011; Freveret al. 2013; Liu et al. 2013, 2014; Schlegelmilchet al. 2014; Gurbuz and Chiquet-Ehrismann 2015). Furthermore, elevated levels of WISP1 and CYR61 in the primary breast cancers are associated with more advanced features (Xie et al. 2001; Chiang et al. 2015). WISP2, which is a transcriptional repressor of genes associated with the epithelial-mesenchymal transition, may act as a dominant-negative regulator of other CCN family members and play an important role in the maintenance of the differentiated phenotype in breast cancer cells, but in contrast, WISP2 is undetectable in more aggressive breast cancer cells (Ferrand et al. 2012; Ji et al. 2014).

The endoplasmic reticulum is the principal intracellular structure responsible for multiple cellular functions including protein folding, maturation and the maintenance of cellular homeostasis. The ER stress is activated by a variety of factors and triggers the unfolded protein response/ER stress, which induce a set of complex intracellular signaling events in the endoplasmic reticulum and restores homeostasis or activates cell death (Moenner et al. 2007; Wang and Kaufman, 2012; Yadav et al. 2014). It is a dynamic intracellular organelle with exquisite sensitivity to alterations in homeostasis, and provides stringent quality control systems to ensure that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are retained and ultimately degraded (Lin et al. 2008). The ER stress is mediated by three sensors and signaling pathways, but IRE1 is a central mediator of the unfolded protein response and an important component of tumor growth. Its blockade leads to a suppression of tumor growth through down-regulation of the angiogenesis and proliferation processes (Drogat et al. 2007; Auf et al. 2010, 2013). The ER stress is recognized as an important determinant of cancer, obesity, and type 2 diabetes and contributes to the expression profile of many regulatory genes resulting in proliferation, apoptosis, angiogenesis, and peripheral insulin resistance (Ozcan et al. 2004; Lin et al. 2008; Minchenko et al. 2013; Clarke et al. 2014; Han and Kaufman 2014; Lee and Ozcan 2014; Manie et al. 2014). Two distinct catalytic domains of the signaling enzyme IRE1 were identified: a serine/threonine kinase and endoribonuclease. The endoribonuclease is responsible for degradation of a specific subset of mRNA and alternative splicing of the XBP1 (X-box binding protein 1) transcription factor mRNA for control of the expression of unfolded protein response-specific genes (Acosta-Alvear et al. 2007; Aragon et al 2009; Hollien et al 2009; Auf et al. 2010; Dejeans et al. 2013; Han et al. 2013; Pluquet et al. 2013).

The ER stress response-signaling pathway is tightly associated with nutrient deprivation and hypoxia and linked to the neovascularization, tumor growth, and cell death processes as well as suppression of insulin receptor signaling through activation of c-Jun N-terminal kinase (JNK) and subsequent serine phosphorylation of IRS1 (Langlais et al. 2011; Lenihan and Taylor 2013; Clarke et al. 2014; Minchenko et al. 2014). The endoplasmic reticulum has an essential position as a signal integrator in the cell and is instrumental in the different phases of tumor progression because the signaling pathways elicited by ER stress sensors have connections with metabolic pathways and with other plasma membrane receptor signaling networks (Bravo et al. 2013; Cao and Kaufman, 2013; Han et al. 2013; Clarke et al. 2014; Manie et al. 2014).

The main goal of this work was to study the role of expression of genes encoding the IGFBPs with different affinity to IGF proteins (IGFBP6, IGFBP7, IGFBP8/CTGF/CCN2, IGFBP9/NOV/CCN3, IGFBP10/CYR61/

CCN1, WISP1/CCN4, and WISP2/CCN5) in U87 glioma cell line and its subline with IRE1 loss of function upon glucose deprivation for evaluation of its possible significance in the control of tumor growth through IRE1 mediated ER stress signaling.

Materials and Methods

Cell lines and culture conditions. The glioma cell line U87 was obtained from ATCC (U.S.A.) and grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's minimum essential medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, U.S.A.) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., U.S.A.), penicillin (100 U/ml; Gibco) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO_2 incubator. In this work, we used two sublines of this glioma cells. One subline was obtained by selection of stable transfected clones with overexpression of vector pcDNA3.1, which was used for creation of dn-IRE1. This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of the effect of glucose deprivation on the expression level of different *IGFBP* and related genes. Second subline was obtained by selection of stable transfected clone with overexpression of IRE1 dominant/negative construct (dn-IRE1) and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme (clone 1C5) (Auf et al. 2010). The expression level of studied genes in these cells was compared with cells, transfected by vector (control 1), but this subline was also used as control 2 for investigation the effect of glucose deprivation on gene expressions under blockade of both enzymatic activities of IRE1. The efficiency of IRE1 suppression in this glioma cell subline has been estimated previously (Auf et al. 2010, 2013; Minchenko et al. 2013) by determining the expression level of the XBP1 alternative splice variant, a key transcription factor in the IRE1 signaling, and the level of the phosphorylated isoform IRE1 using cells treated by tunicamycin (0.01 mg/ml during 2 h). Both used in this study sublines of glioma cells are grown with the addition of geneticin (G418) while these cells carrying empty vector pcDNA3.1 or dn-ERN1 construct.

Glucose deprivation condition was created by changing the complete DMEM medium into culture plates on DMEM medium without glucose (Gibco) and plates were exposed to this condition for 16 h.

RNA isolation. Total RNA was extracted from glioma cells using the Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA pellets was 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.

Reverse transcription and quantitative PCR analysis. The expression levels of insulin-like growth factor binding proteins-6, -7, -9 and -10 (IGFBP6, IGFBP7, IGFBP8/CTGF, IGFBP9/NOV, and IGFBP10/CYR61) and WNT1 inducible signaling pathway protein-1 and -2 (WISP1 and WISP2) mRNA's as well as ACTB mRNA were measured in control U87 glioma cells and cells with a deficiency of IRE1 by quantitative polymerase chain reaction in real-time using „Mx 3000PQPCR" (Stratagene, La Jolla, CA, U.S.A.) and SYBRGreenMix (ABgene, Thermo Fisher Scientific, Epsom, Surrey, UK). Quani Tect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis as described previously (Minchenko et al. 2012). Polymerase chain reaction was performed in triplicate.

For amplification of IGFBP6 cDNA we used forward (5'–gctgttgcagaggagaatcc–3'and reverse (5'–ggtagaagcctcgatggtca–3') primers. The nucleotide sequences of these primers correspond to sequences 397 – 416 and 655 – 636 of human IGFBP6 cDNA (GenBank accession number NM_002178). The amplification of IGFBP7cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward–5'–agctgtgaggtcatcggaat–3' and reverse–5'–tatagctcggcaccttcacc–3'. The nucleotide sequences of these primers correspond to sequences 572 – 591 and 882 – 863 of human IGFBP7 cDNA (GenBank accession number NM_001553). For amplification of IGFBP8/CTGF/CCN2 cDNA, we used forward (5'–ttccagagcagctgcaagta–3') and reverse (5'–ccaggcagttggctctaatc–3') primers. The nucleotide sequences of these primers correspond to sequences 552 – 571 and 807 – 788 of human IGFBP8 cDNA (GenBank accession number NM_001901). The amplification of IGFBP9/NOV/CCN3 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward–5'–gcgaagaaagtctcgtttgg–3' and reverse–5'–acaccagacagcatgagcag–3'. The nucleotide sequences of these primers correspond to sequences 176 – 195 and 420 – 401 of human IGFBP9 cDNA (GenBank accession number NM_002514). For amplification of IGFBP10/CYR61/CCN1 cDNA, we used forward (5'–ctccctgtttttggaatgga–3') and reverse (5'–tggtcttgctgcatttcttg–3') primers**.** The nucleotide sequences of these primers correspond to sequences 852 – 871 and 1092 – 1073 of human IGFBP10 cDNA (GenBank accession number NM_001554). The amplification of WNT1 inducible

signaling pathway protein 1 (WISP1), also known as CCN4, cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward–5'–gactttaccccagctccact–3' and reverse–5'–gtagtcacagtagaggcccc–3'. The nucleotide sequences of these primers correspond to sequences 203 – 222 and 415 – 396 of human WISP1 cDNA (GenBank accession number NM_003882). For amplification of WISP2/CCN5 cDNA, we used forward (5'–ctgtatcgggaaggggagac–3') and reverse (5'–gggaagagacaaggccagaa–3') primers. The nucleotide sequences of these primers correspond to sequences 463 – 482 and 709 – 690 of human WISP2 cDNA (GenBank accession number NM_003881). The amplification of ACTB (beta-actin) cDNA was performed using forward–5'–ggacttcgagcaagagatgg–3' and reverse–5'–agcactgtgttggcgtacag–3' primers. The primers nucleotide sequences correspond to 747 – 766 and 980 – 961 of human ACTB cDNA (GenBank accession number NM_001101). The expression of beta-actin mRNA was used as control of analyzed RNA quantity. The primers were received from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Quantitative PCR analysis was performed using a special computer program "Differential expression calculator" and statistical analysis, as described previously (Bochkov et al 2006). The values of *IGFBP6*, *IGFBP7*, *IGFBP8/CTGF*, *IGFBP9/NOV*, *IGFBP10/CYR61*, *WISP1*, and *WISP2* gene expressions were normalized to the expression of beta-actin mRNA and represent as percent of control (100%). All values are expressed as mean ± SEM from triplicate measurements performed in four independent experiments. The amplified DNA fragments were also analyzed on 2% agarose gel and that visualized by SYBR* Safe DNA Gel Stain (Life Technologies, Carlsbad, CA, U.S.A.).

Results

Effect of glucose deprivation and inhibition of IRE1 on the expression of *IGFBP6* and *IGFBP7* genes in glioma cells. To investigate a possible role of endoplasmic reticulum stress signaling mediated by IRE1 in the expression of IGFBP genes *IGFBP6* and *IGFBP7* and its sensitivity to glucose deprivation we studied the effect of glucose deprivation on these genes expression in glioma cells with normal IRE1 function (control glioma cells) and cells without both enzymatic activities of this signaling enzyme. As shown in Fig. 1, the expression level of IGFBP6 mRNA is significantly down-regulated in U87 glioma cells (2.2 times) in comparison with the control cells (control 1). At the same time, inhibition of IRE1 by dn-IRE1 strongly up-regulated the expression level of IGFBP7 mRNA (2.9 times) in glioma cells in comparison with the control cells (Fig. 2). Further, we tested the sensitivity of the genes expression to glucose deprivation and the possible role of ERN1 in the regulation of this sensitivity. We found that expression of *IGFBP6* gene is resistant to glucose deprivation in glioma cells both with and without IRE1 signaling enzyme function, in comparison with corresponding controls (control 1 and 2) (Fig. 1). At the same time, low but statistically significant changes were found in *IGFBP7* gene expression in both types of glioma cells treated by glucose deprivation as compare to corresponding controls (-16% for control cells and -38% for IRE1 knocked-down cells; Fig. 2).

Expression of *IGFBP8/CTGF*, *IGFBP9/NOV* and *IGFBP10/CYR61* genes in control glioma cells and cells without IRE1 function upon glucose deprivation. We investigated the cysteine-rich regulatory proteins IGFBP8/CTGF/CCN2, IGFBP9/NOV/CCN3, and IGFBP10/CYR61/CCN1, which are associated with the extracellular matrix and play an important role in cancer development by regulation of angiogenesis, cell

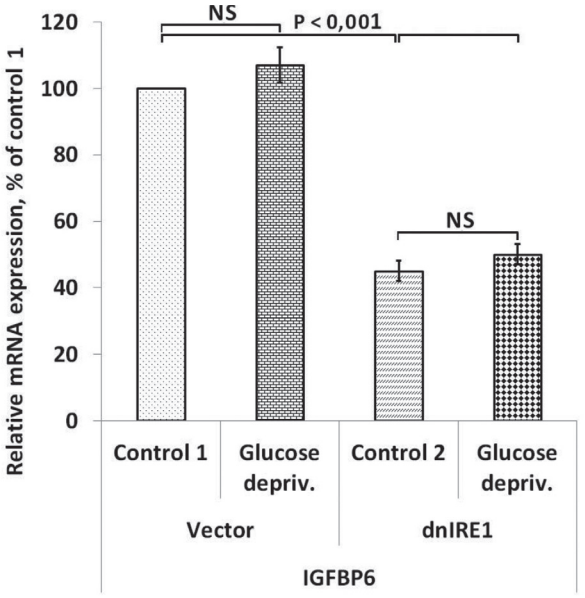

Fig. 1. Effect of glucose deprivation on the expression level of insulin-like growth factor binding protein 6 (IGFBP6) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP6 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

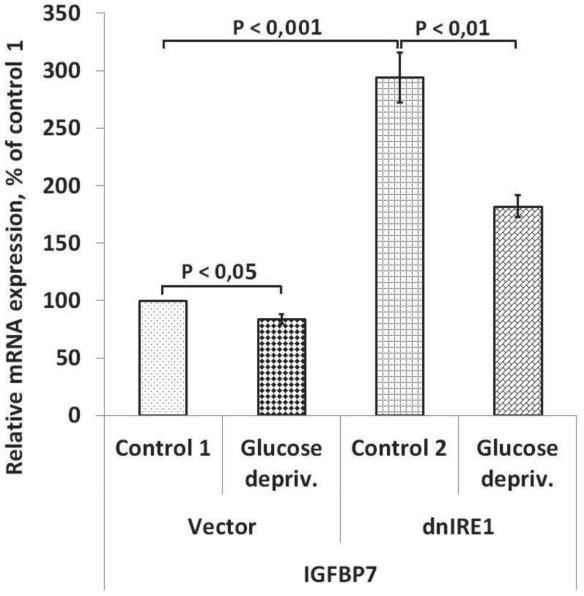

Fig. 2. Effect of glucose deprivation on the expression level of insulin-like growth factor binding protein 7 (IGFBP7) mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP7 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

migration, and proliferation. As shown in Figs. 3, 4, and 5, the expression both *IGFBP8/CTGF*, *IGFBP9/NOV* and *IGFBP10/CYR61* genes are affected by inhibition of IRE1 signaling enzyme function in gene-specific manner. Thus, inhibition of IRE1 enzyme function by dn-IRE1 is strongly down-regulated the expression level of IGFBP9/NOV mRNA (4.8 times), but significantly up-regulated the expression of *IGFBP8/CTGF* (5.4 times) and *IGFBP10/CYR61* (1.5 times) genes in glioma cells in comparison with control glioma cells. Thereafter, we tested how glucose deprivation affects these IGFBP genes and how IRE1 inhibition modulates the effect of glucose deprivation on the expression of *IGFBP8/CTGF*, *IGFBP9/NOV* and *IGFBP10/CYR61* genes. As shown in Fig. 3, glucose deprivation leads to significant up-regulation of IGFBP8/CTGF mRNA level (+36%), but inhibition of IRE1 induces reverse changes in this gene expression upon glucose deprivation (-31%). Stronger up-regulation was shown for *IGFBP9/NOV* gene: 1.9 times in control glioma cells as compared to control 1 and 3 times in cells without IRE1 signaling enzyme function (Fig. 4). At the same time, no significant changes were found in the expression of *IGFBP10/CYR61* gene

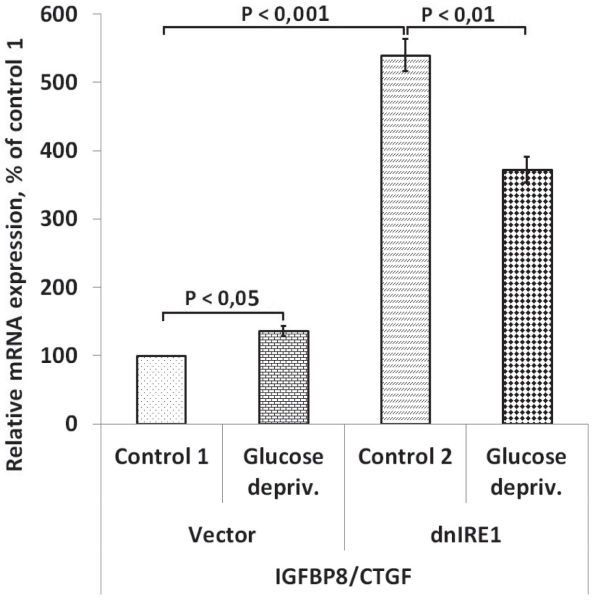

Fig. 3. Effect of glucose deprivation on the expression level of insulin-like growth factor binding protein 8 (IGFBP8), also known as CTGF (connective tissue growth factor) and CCN2 (CCN family member 2), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP8 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

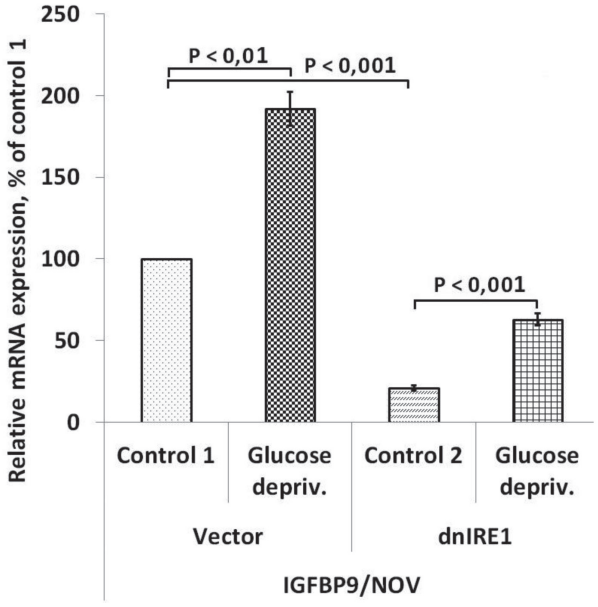

Fig. 4. Effect of glucose deprivation on the expression level of insulin-like growth factor binding protein 9 (IGFBP9), also known as NOV (nephroblastoma overexpressed) and CCN3 (CCN family member 3), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP9 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

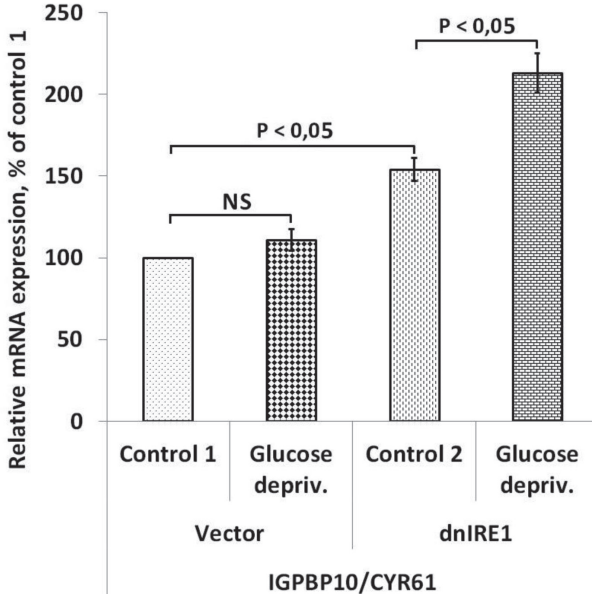

Fig. 5. Effect of glucose deprivation on the expression of insulin-like growth factor binding protein 10 (IGFBP10), also known as CYR61 (cysteine-rich angiogenic inducer 61) and CCN1 (CCN family member 1), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of IGFBP10 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control 1 (100%); n = 4.

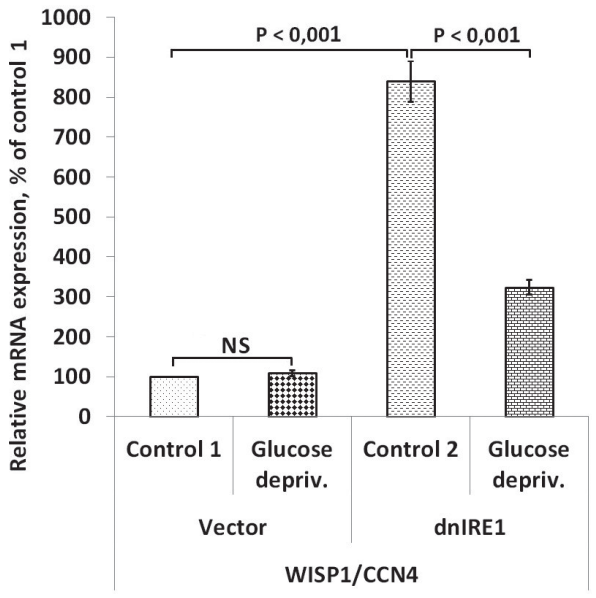

Fig. 6. Effect of glucose deprivation on the expression of WNT1 inducible signaling pathway protein 1 (WISP1), also known as CCN4 (CCN family member 4), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of WISP1 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control1 (100%); n = 4.

in control glioma cells upon glucose deprivation, but inhibition of IRE1 induced sensitivity of this gene expression to glucose deprivation (Fig. 5). Thus, glucose deprivation enhanced *IGFBP10/CYR61* gene expression (+38%) in glioma cells without IRE1 signaling enzyme function.

Inhibition of IRE1 enhances expression of *WISP1* and *WISP2* genes and modifies its sensitivity to glucose deprivation. Additionally, we studied the effect of IRE1 inhibition on the expression of WNT1 inducible signaling pathway protein-1 and -2 (WISP1 and WISP2), which have IGF binding domain and are members of CCN family proteins as well as IGFBP9/NOV/CCN3 and IGFBP10/CYR61/CCN1. As shown in Fig. 6 and Fig. 7, inhibition of IRE1 by dn-IRE1 strongly up-regulates the expression of *WISP1/CCN4* and *WISP2CCN5* genes in glioma cells, indicating for more significant changes for *WISP1/CCN4* gene (8.4 times) as *WISP2/CCN5* one (3.6 times).

Next, we examined *WISP1* gene expression in glioma cells upon glucose deprivation condition. As shown in Fig. 6, this gene expression was resistant to glucose deprivation in cells with native IRE1, but inhibition of IRE1 signaling enzyme function lead to a strong down-regulation of *WISP1* gene expression (2.6 times). At the same time, exposure of glioma cells to glucose deprivation induces the expression of *WISP2* gene in both types of glioma cells: +45% in control glioma cells and 30% in cells with a deficiency of IRE1 enzyme function (Fig. 7).

Additionally, we analyzed the effect of glucose deprivation on the expression level of IGFBP7, IGFBP9/NOV/CCN3, and WISP2/CCN5 mRNA in glioma cells with intact and inhibited IRE1 enzyme function in conditions when both controls (control 1 and control 2) were considered as 100% for the possibility to more precisely clarify the differences in the sensitivity of these gene expressions to glucose deprivation with respect to inhibition of IRE1. As showed in Fig. 8, there are statistically significant differences in the expression levels of IGFBP7, IGFBP9/NOV, and WISP2 mRNA in control glioma cells and cells without IRE1 function exposure upon glucose deprivation: 2.4 times for IGFBP7, 1.6 times for IGFBP9/NOV and 1.5 times for WISP2.

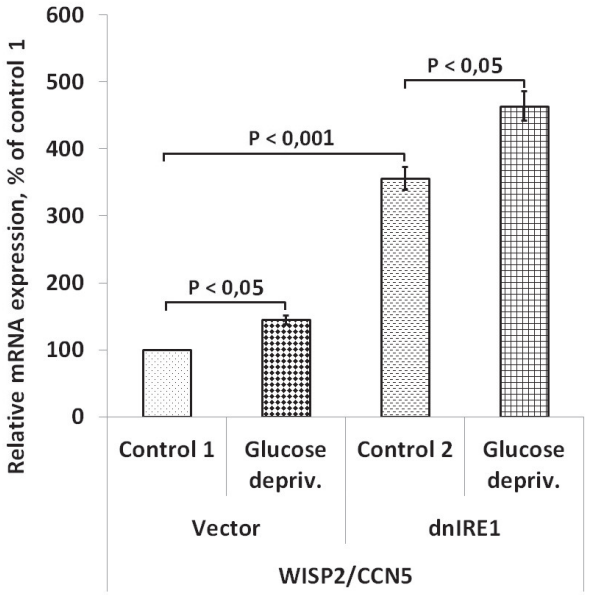

Fig. 7. Effect of glucose deprivation on the expression of WNT1 inducible signaling pathway protein 2 (WISP2), also known as CCN5 (CCN family member 5), mRNA in control U87 glioma cells (Vector) and cells with a blockade of the IRE1 by dn-IRE1 (dn-IRE1) measured by qPCR. Values of WISP2 mRNA expressions were normalized to beta-actin mRNA level and represent as percent for control1 (100%); n = 4.

Discussion

The growing tumor requires ER stress as well as nutrient deprivation, which initiate the ER stress for own neovascularization and growth, for apoptosis inhibition (Drogat et al. 2007; Auf et al. 2010). Cell proliferation is strongly dependent upon glycolysis and glucose level because there is a molecular connection between cell cycle progression and the provision of nutrients essential for this purpose (Colombo et al. 2011). The ER has an important position as a signal integrator in both normal and malignant cells because the ER stress signaling pathways have connections with other plasma membrane receptor signaling networks involved in numerous metabolic pathways (Bravo et al. 2013; Minchenko et al. 2013; Manie et al. 2014). It is known that the complete blockade of the activity of IRE1 signaling enzyme in glioma cells had anti-tumor effects (Auf et al. 2010, 2013; Minchenko et al. 2014, 2015a). In this work, we studied the expression of genes encoded different IGFBPs in glioma cells with inhibition of IRE1 signaling enzyme function upon glucose deprivation condition for evaluation of possible significance of these genes in the control of glioma growth through ER stress signaling mediated by IRE1 and glucose deprivation.

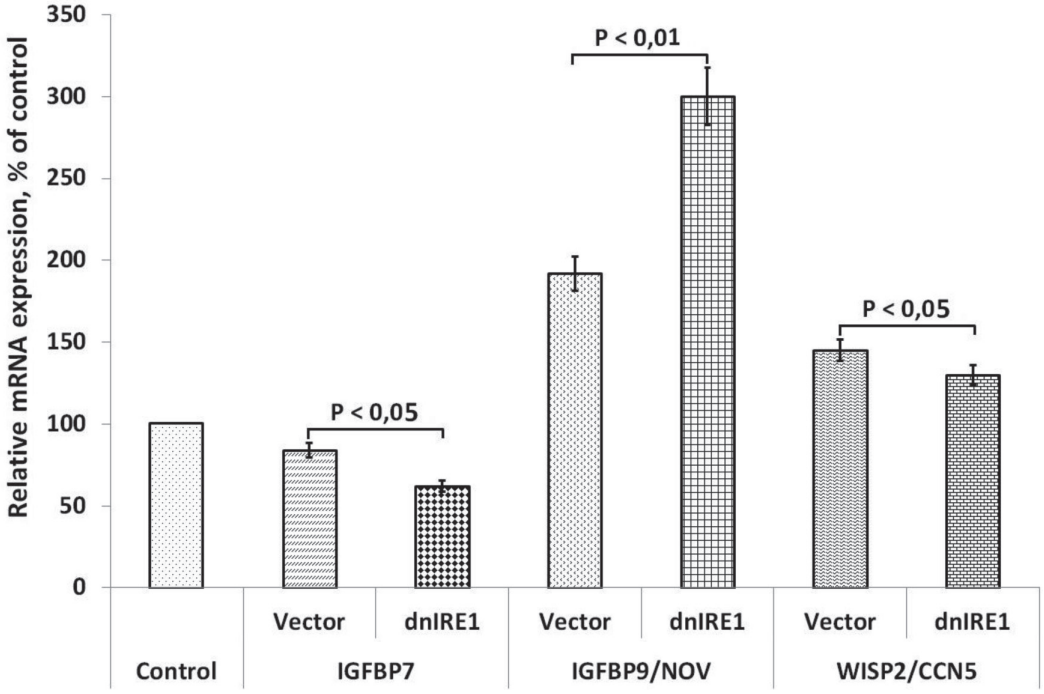

Fig. 8. Inhibition of IRE1 modifies the effect of glucose deprivation condition on the expression of IGFBP7, IGFBP9/NOV, and WISP2 mRNA in glioma cells (by qPCR). The mRNA expression values were normalized to beta-actin mRNA expression and represent as percent of corresponding control (both controls is accepted as 100%); mean ± SEM; n = 4.

Results of this study demonstrate that inhibition of IRE1 enzyme function significantly decreased the expression of *IGFBP6* and *IGFBP9/NOV* genes indicating its participation in IRE1 mediated network of unfolded protein response. It is possible that decreased expression of *IGFBP6* and *IGFBP9/NOV* genes in glioma cells without both enzymatic activities of IRE1 will contribute to the suppression of glioma growth from cells with IRE1 knockdown (Drogat et al. 2007; Auf et al. 2010, 2013). These results conform the data that *IGFBP6* and *IGFBP9/NOV* have mainly pro-proliferative functions through interaction with different proteins and signaling pathways and are overexpressed in various malignant tumors (Micutkova et al. 2011; Bach et al. 2013; Wagener et al. 2013; Baxter 2014; Liu et al. 2015a).

The IGFBPs bind and regulate the availability of both IGFs with different affinity and inhibit or stimulate the growth promoting effects of the IGFs through IGF/INS receptors and through many other signaling pathways and regulate cell proliferation and survival as well as angiogenesis and cancer cell migration (Weroha and Halushka 2012; Grkovic et al. 2013; Wagener et al. 2013; Baxter 2014; Pan et al. 2014; Singh et al. 2014; Zhu et al. 2014; Chen et al. 2015). In this study, we showed that inhibition of IRE1 signaling, which is a central mediator of the unfolded protein response and an important component of malignant tumor growth, is strongly up-regulate the expression level of *IGFBP7* gene in glioma cells and that this induction of IGFBP7 may contribute to suppression of IRE1 knockdown cell proliferation, because this protein acts as a tumor suppressor (Zhu et al. 2014). Moreover, effect of IGFBP7 on cell proliferation, apoptosis, and senescence is realized through both insulin/IGF-dependent and -independent mechanisms (Zhu et al. 2014).

We also find a significant up-regulation of *IGFBP8/CTGF/CCN2*, *IGFBP10/CYR61/CCN1*, *WISP1/CCN4*, and *WISP2/CCN5* gene expressions in glioma cells after inhibition of IRE1 signaling, which supports the IRE1-mediated mechanisms of the expression of both these genes. Furthermore, up-regulation of the expression of these multifunctional proteins in glioma cells after inhibition of IRE1 can also contribute to the suppression of these glioma cells proliferation, because there is data indicating that elevated expression of CTGF, WISP-1, and CYR61 in primary breast cancers associated with more advanced features (Xie et al. 2001) and that CTGF binds VEGF and suppresses VEGF-induced angiogenesis (Inoki et al. 2011). It is possible that IGFBP10/CYR61/CCN1 can suppress hepatic carcinogenesis by inhibiting compensatory proliferation (Chen et al. 2015), although its role in tumor growth is not well established yet. At the same time, increased level of CYR61/CCN1 as well as decreased level of NOV/CCN3 may have a relation to increased metastasis of glioma cells without IRE1 function (Auf et al. 2010), because CYR61/CCN1 is responsible for induction of the tumor epithelial-mesenchymal transition, invasion, and metastasis (Hou et al. 2014; Liu et al. 2015b) as well as inhibition of NOV levels promotes the invasive phenotype of metastatic cancer cells (Dobson et al. 2014). Furthermore, elevated levels of WISP1 and CYR61 in primary breast cancers are associated with more advanced features (Xie et al. 2001; Chiang et al. 2015). WISP2 is a transcriptional repressor of genes associated with the epithelial-mesenchymal transition; it is undetectable in more aggressive breast cancer cells (Ferrand et al. 2012; Ji et al. 2014). Thus, up-regulation of WISP1 and WISP2 gene expression in glioma cells after inhibition of IRE1 can also contribute to the suppression of these glioma cells proliferation. Thus, our results demonstrate that all of the genes studied are ER stress responsive and consequently have a pivotal role in the control of cell proliferation as well as metastasis, but the mechanisms of activation or suppression of these gene expressions upon inhibition of IRE1 differs and warrant further investigation.

The investigation of the expression of different genes of IGFBP and CCN families in glioma cells upon glucose deprivation in respect to inhibition of IRE1 signaling is very important for understanding of malignant tumor growth mechanisms, because glucose play essential role in the cell cycle control as that tumor progression (Colombo et al. 2011; Huber et al. 2013). We showed that glucose deprivation condition leads to up-regulation of the expression of *IGFBP8/CTGF/CCN2*, *IGFBP9/NOV/CCN1*, and *WISP2/CCN5* genes and down-regulation of *IGFBP7* gene in control glioma cells. At the same time, the expression of *IGFBP6*, *IGFBP10*, and *WISP1* genes was resistant to glucose deprivation in this type of glioma cells. However, inhibition of IRE1 in glioma cells modifies the sensitivity of most studied gene expressions to glucose deprivation condition and enhances effect of IRE1 inhibition on *IGFBP10/CYR61/CCN1* and *WISP2* gene expressions as well as reduces this effect on *IGFBP7*, *NOV/CCN3* and *WISP1* genes. Our results are consistent with the data of Huber et al. (2013) that glucose shortage is associated with malignant progression through the ER unfolded protein response, but the mechanism how malignant cells cope with potentially lethal metabolic stress induced by glucose deprivation remains poorly understood.

The changes observed in the above studied IGFBP gene expressions correlate well with slower cell proliferation in cells harboring dn-IRE1, attesting to the fact that ER stress is a necessary component of malignant tumor growth, cell survival, and metastasis. Moreover, the expression of IGFBP genes upon glucose deprivation is significantly depended on IRE1 signaling enzyme function. However, the detailed molecular mechanisms of this regulation are complex and warrants further study.

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