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THE EXPRESSION OF CDC20, CDC42 AND CDC42-EFFECTOR GENES IN U87 GLIOMA CELLS WITH ERN1 SIGNALING ENZYME LOSS OF FUNCTION: EFFECT OF HYPOXIA

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A set of complex intracellular signaling events known as the unfolded protein response, mediated predominantly by endoplasmic reticulum – nuclei-1 (ERN1) signaling enzyme, is induced in cancer cells via hypoxia and ischemia and is necessary for neovascularization and proliferation processes. A lot of cell division cycle proteins, cyclins, cyclin-dependent kinases and its inhibitors are the components of endoplasmic reticulum stress system as well as participate in the control of cell cycle and proliferation processes. We have studied the expression of genes which are participate in the control of cell cycle and proliferation in glioma cell line U87 and its ERN1-deficient subline in hypoxic conditions. It was shown that the blockade of ERN1 leads to decrease the expression levels of cell division cycle-20 (CDC20) as well as CDC42-effectors CDC42EP4 and PAK4 mRNA expressions, but increases the levels of CDC42EP2, CDC42EP3 and CDC42SE1 mRNA expressions. However, the expression level of CDC42 mRNA did not change significantly in glioma cells with ERN1 loss of function. Moreover, we have shown that hypoxia does not affect the expression of CDC42 as well as CDC42-effectors CDC42SE1 and CDC42EP3 mRNA expressions, but increases PAK4 and CDC42EP2 and decreases CDC20 genes in cells with functional ERN1 (control glioma cells). However, the hypoxic regulation of CDC42 as well as some CDC42-effectors gene expressions significantly depends from ERN1 signaling enzyme function because its blockade leads to increase of CDC42 and decrease of CDC42EP2 gene expressions by hypoxia. Thus, results of this study clearly demonstrated that the expression levels of most tested genes encoded CDC42 and its effectors is mostly depend from ERN1 signaling enzyme function and possibly participate in cell adaptive response to endoplasmic reticulum stress associated with hypoxia.

Key words: mRNA expression, CDC20, CDC42, CEP2, CEP3, CEP4, PAK4, ERN1 knockdown, glioma cells, hypoxia.

Introduction. Hypoxia as well as nutrient deprivation conditions are important factors of malignant tumor growth. Hypoxia and many other factors induce the endoplasmic reticulum stress and a large number of different gene expressions which enhance metabolism and proliferative processes (Johnson et al., 2008; Denko, 2008). The endoplasmic reticulum stress is associated with unfolded protein response and accumulation of unfolded proteins in the endoplasmic reticulum (Zhang, Kaufman, 2004). This adaptive response is activated upon the accumulation of misfolded proteins in the endoplasmic reticulum and is mediated by three endoplasmic reticulum-resident sensors named PERK (PRK-like ER kinase), IRE1/ERN1 (Inositol Requiring Enzyme-1/Endoplasmic Reticulum to Nuclei-1) and ATF6 (Activating Transcription Factor 6), however, ERN1 is the dominant sensor (Bi et al., 2005; Aragón et al., 2009; Fels, Koumenis, 2006; Korennykh et al., 2009). Activation of the unfolded protein response tends to limit the de novo entry of proteins in to the endoplasmic reticulum and facilitate both the endoplasmic reticulum protein folding and degradation to adapt cells for survival or, alternatively, to enter cell death programs through endoplasmic reticulum-associated machineries (Moenner et al., 2007; Romero-Ramirez et al., 2004; Acosta-Alvear et al., 2007; Hollien et al., 2009). As such, it participates in the early cellular response to the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum, occurring under both physiological and pathological situations (Zhang, Kaufman, 2004; Moenner et al., 2007).

The bifunctional signaling enzyme ERN1 has two distinct catalytic domains: a serine/threonine kinase and an endoribonuclease. Both domains are contributed to ERN1 signalling. The ERN1-associated kinase autophosphorylates and dimerizes this enzyme in membrane, leading to the activation of its endoribonuclease domain, degradation of a specific subset of mRNA and initiation of the pre-XBP1 (X-box binding protein 1) mRNA splicing (Romero-Ramirez et al., 2004; Acosta-Alvear et al., 2007). Mature XBP1 mRNA splice variant encodes a transcription factor that stimulates the expression more than five hundreds of unfolded protein response-specific genes (Acosta-Alvear et al., 2007; Hollien et al., 2009). Moreover, XBP1s has several additional functions, especially in the regulation of glucose homeostasis

(Lee et al., 2011; Zhou et al., 2011). Thus, the p38 MAP kinase phosphorylates the spliced form of XBP1 and enhances its nuclear migration. Moreover, the regulatory subunits of phosphatidyl inositol 3-kinase interact with XBP1 and also increase its nuclear translocation (Park et al., 2010). Zhou et al. (Zhou et al., 2011) shown that XBP1s interacts with the Forkhead box O1 (FOXO1) transcription factor and directs it toward proteasome-mediated degradation. At the same time, it was shown that a kinase inhibitor activates the ERN1 endoribonuclease to confer cytoprotection against ER stress. It is possible that this activation of the ERN1 endoribonuclease is a result of its interaction with other sensor-signalling systems of endoplasmic reticulum stress.

The endoplasmic reticulum stress response-signalling pathway is associated with hypoxia and linked to the proliferation process and tumor growth because the complete blockade of ERN1 signal transduction pathway has anti-tumor effects in lung cancer and glioma cells (Romero-Ramirez et al., 2004; Drogat et al., 2007; Auf et al., 2010). Malignant gliomas are highly aggressive tumors and are characterized by marked angiogenesis and extensive tumor cell invasion into the normal brain parenchyma (Moenner et al., 2007). By itself, the invasive growth pattern prevents complete surgical resection and leads to local recurrences. The very poor prognosis and the moderate efficacy of conventional clinical approaches therefore underline the need for new therapeutic strategies. Hypoxia is associated to glioma development and locally induces an adaptive response which confers to tumor cells an enhanced survival and a more agressive behaviour (Moenner et al., 2007). A better knowledge of tumor responses to ischemia is required to elaborate therapeutical strategies of cell sensibilization and angiogenesis inhibition, based on the blockade of survival mechanisms.

Cell division cycle regulatory proteins play an important role in glycolysis and tumor growth. Thus, cell division cycle 42 (CDC42) is a small Rho GTPase which regulates the formation of F-actin-containing structures through its interaction with the downstream effector proteins and is important for cancer growth because downregulation of CDC42 and CDK6 (cyclin-dependent kinase 6) by microRNA 137 induces G1 cell cycle arrest in lung cancer cells as well as promotes transendothelial migration of cancer cells through beta1 integrin (Zhu et al., 2013; Reymond et al., 2012). Moreover, the Rho family GTPases CDC42 and Rac1 may contribute to the hypoxia-mediated angiogenesis because both are involved in the hypoxia-induced production of angiogenesis-promoting factors and tumor suppressors (Xue et al., 2006). CDC42 effector protein (Rho GTPase binding) 2, 3 and 4 (CDC42EP2, CDC42EP3 and CDC42EP4) are members of the Borg family of CDC42 effector proteins which bind to, and negatively regulate the function of, CDC42 and probably involved in the organization of the actin cytoskeleton (Lin, Chuang, 2012).

PAK4 is a serine/threonine p21-activating kinase that links Rho GTPases to cytoskeleton reorganization and nuclear signaling. It has been implicated in a wide range of biological activities, because plays a role in a variety of different signaling pathways including cell migration, growth, proliferation or cell survival by phosphorylation of integrin beta5 and BCL2 antagonist of cell death BAD (Wang et al., 2012; Zhang et al., 2012; Zhang et al., 2002).

The main goal of this study was investigation the role of the expression of genes related to the control of cell cycle and proliferation (CDC42, CDC20, CDC42SE1, CDC42EP2, CDC42EP3, CDC42EP4 and PAK4) in glioma U87 cells with ERN1 signaling enzyme loss of function and its regulation by hypoxia.

Materials and Methods. The glioma cell line U87 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) and streptomycin (0.1 mg/ml; Gibco) at 37^{o}C in a 5% CO_2 incubator. In this work we used two sublines of this glioma cell line. One subline was obtained by selection of stable transfected clones with overexpression of vector (pcDNA3.1), which was used for creation of dominant-negative constructs (dnERN1). This untreated subline of glioma cells (control glioma cells) was used as control 1 in the study of effects of hypoxia on the expression level of CDC42 and related genes. Second subline was obtained by selection of stable transfected clones with overexpression of dnERN1 and has suppressed both protein kinase and endoribonuclease activities of this signaling enzyme. These cells were obtained from prof. M. Moenner (France) (Moenner et al., 2007; Drogat et al., 2007). The expression level of CDC42 and related 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 hypoxia on the expression level of these genes under blockade ERN1 function. Hypoxic conditions were created in special incubator with 3 % oxygen and 5 % carbon dioxide levels; culture plates with complete DMEM were exposed to these conditions for 16 hrs.

The suppression level of ERN1 both enzymatic activity in glioma cells that overexpress a dominant-negative construct of endoplasmic reticulum–nuclei-1 (dnERN1) was previously shown by analysis of the expression of XBP1 alternative splice variant (XBP1s), a key transcription factor in ERN1 signaling, and phosphorylated isoform ERN1 using cells

treated by tunicamycin (0.01 mg/ml during 2 hours) (Minchenko et al., 2012).

Total RNA was extracted from glioma cells as described (Minchenko et al., 2012). RNA pellets were washed with 75 % ethanol and dissolved in nuclease-free water. For additional purification RNA samples were precipitated with 95 % ethanol and redissolved again in nuclease-free water.

QuaniTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis as described previously (Minchenko et al., 2012).

The expression levels of CDC24, CDC20, CDC42SE1, CDC42EP2, CDC42EP3, CDC42EP4 and PAK4 mRNA were measured in glioma cell line U87 and its sublines (clone 1C5) by real-time quantitative polymerase chain reaction of cDNA using „Mx 3000P QPCR" (Stratagene, USA) and SYBRGreen Mix (AB gene, Great Britain). Polymerase chain reaction was performed in triplicate. For amplification of cell division cycle 24 cDNA we used forward (5'–tccccatctggtgctcttag –3' and reverse (5'– tggcaaacaaatgtccttga –3') primers. The nucleotide sequences of these primers correspond to sequences 1312 – 1331 and 1515 – 1496 of human CDC24 cDNA (GenBank accession number NM_001791). The size of amplified fragment is 204 bp. The amplification of cell division cycle 20 (CDC20) cDNA was performed using forward primer (5'– tccaaggttcagaccactcc–3') and reverse primer (5'– ctggcagggaaggaatgtaa –3'). These oligonucleotides correspond to sequences 294 – 313 and 618 – 599 of human CDC20 cDNA (GenBank accession number NM_001255). The size of amplified fragment is 325 bp.

The amplification of CDC42 small effector 1 (CDC42SE1 or SPEC1) cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– gtccaaacaccagggaaaga –3' and reverse – 5'– ctcctgaactgcacctgtca –3'. The nucleotide sequences of these primers correspond to sequences 366 – 385 and 617 – 598 of human CDC42SE1 cDNA (GenBank accession number NM_020239). The size of amplified fragment is 252 bp. For amplification of CDC42 effector protein 2 (CDC42EP2) cDNA we used forward (5'– tgtccagctcctgagacctt –3' and reverse (5'– aggacggcaaactcttctga –3') primers. The nucleotide sequences of these primers correspond to sequences 84 – 103 and 251 – 232 of human CDC42EP2 cDNA (GenBank accession number NM_006779). The size of amplified fragment is 168 bp. The amplification of CDC42EP3 cDNA for real time RCR analysis was performed using two oligonucleotides primers: forward – 5'– tttgtcctggttgtggtcaa –3' and reverse – 5'– agggccactttcttcacaga –3'. The nucleotide sequences of these primers correspond to sequences 366 – 385 and 617 – 598 of human CDC42EP3 cDNA (GenBank accession number NM_006449). The size of amplified fragment is 196 bp. For amplification of CDC42 effector protein 4 (CDC42EP4) cDNA we used forward (5'– ccaatcctcaagcaactggt –3' and reverse (5'– aggagactgcgtttggaaga –3') primers. The nucleotide sequences of these primers correspond to sequences 228 – 247 and 460 – 441 of human CDC42EP4 cDNA (GenBank accession number NM_012121). The size of amplified fragment is 233 bp.

The amplification of p21 protein (Cdc42/Rac)-activated kinase 4 (PAK4) cDNA for real time RCR analysis was performed using two oligonucleotide primers: forward – 5'– gcgagtatcccatgagcagt –3' and reverse – 5'– aactccatgaccacccagag –3'. The nucleotide sequences of these primers correspond to sequences 1126 – 1145 and 1425 – 1406 of human PAK4 cDNA (GenBank accession number NM_005884). The size of amplified fragment is 300 bp. The amplification of beta-actin (ACTB) cDNA was performed using forward - 5'– ggacttcgagcaagagatgg –3' and reverse - 5'– agcactgtgttggcgtacag –3' primers. These primers 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. The primers were received from "Sigma" (USA).

An analysis of quantitative PCR was performed using special computer program "Differential expression calculator" and statistical analysis – in Excel program. The values of CDC24, CDC20, CDC42SE1, CDC42EP2, CDC42EP3, CDC42EP4 and PAK4 mRNA 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 4 independent experiments.

Results and Discussion. As shown in fig. 1, the suppression of both enzymatic functions of sensor and signaling enzyme ERN1, the major component of endoplasmic reticulum stress signaling, leads to 2 fold decrease of the expression of gene encoding of cell division cycle-20 (CDC20), but does not change significantly CDC42 mRNA expression. At the same time, the expression level of CDC42-effectors (CDC42SE1, CDC42EP2 and CDC42EP3) is increased in glioma U87 cells with suppressed ERN1 signaling enzyme function, being significant higher for CDC42EP3 gene, as compared to control glioma cells which was transfected with vector pcDNA3.1 (Fig. 2 and 3). However, the expression of two other CDC42-effectors (CDC42EP4 and PAK4) is decreased (close to 2 fold) in glioma cells with ERN1 loss of function (Fig. 3 and 4).

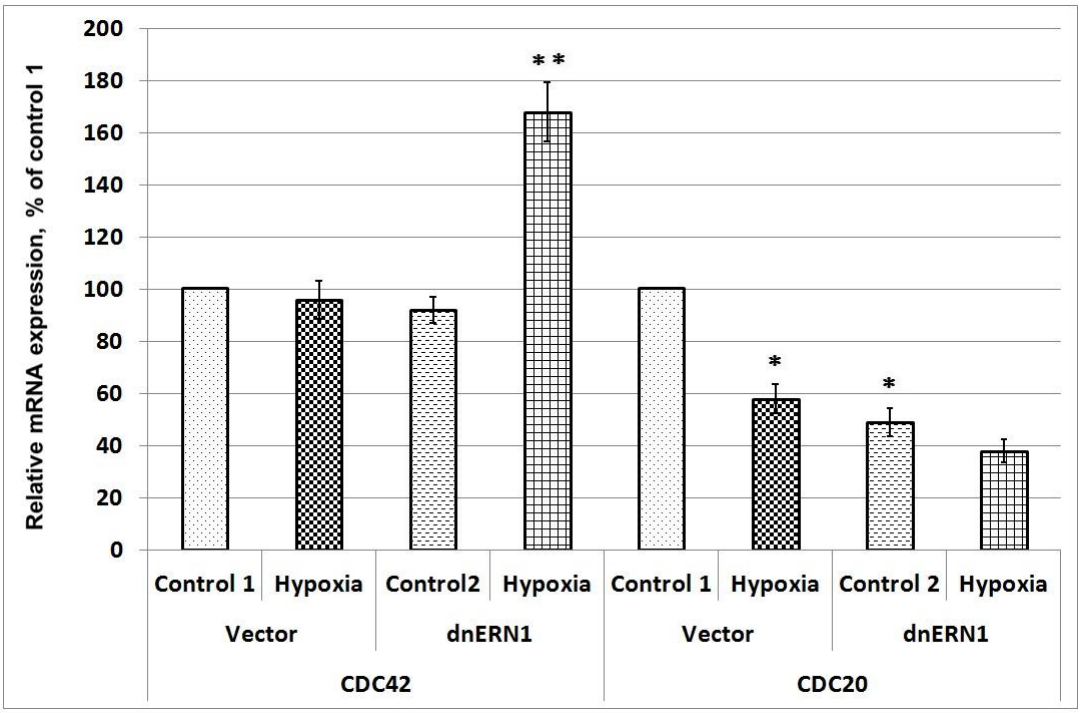

Fig. 1. Effect of hypoxia on the expression level of CDC42 and CDC20 in U87 glioma cells, transfected with vector pcDNA3.1 (Control) and cells, stable transfected with dominant/negative constructs of ERN1 signaling enzyme (dnERN1) into vector pcDNA3.1, measured by quantitative real-time PCR. *The level of these mRNA expressions was normalized to the expression of beta-actin. The changes in the expression of CDC42 and CDC20 mRNA in cells, transfected with dnERN1, were compared with control (100 %); n = 4; * – P < 0,05 as compared to control 1, ** – P < 0,05 as compared with control 2.*

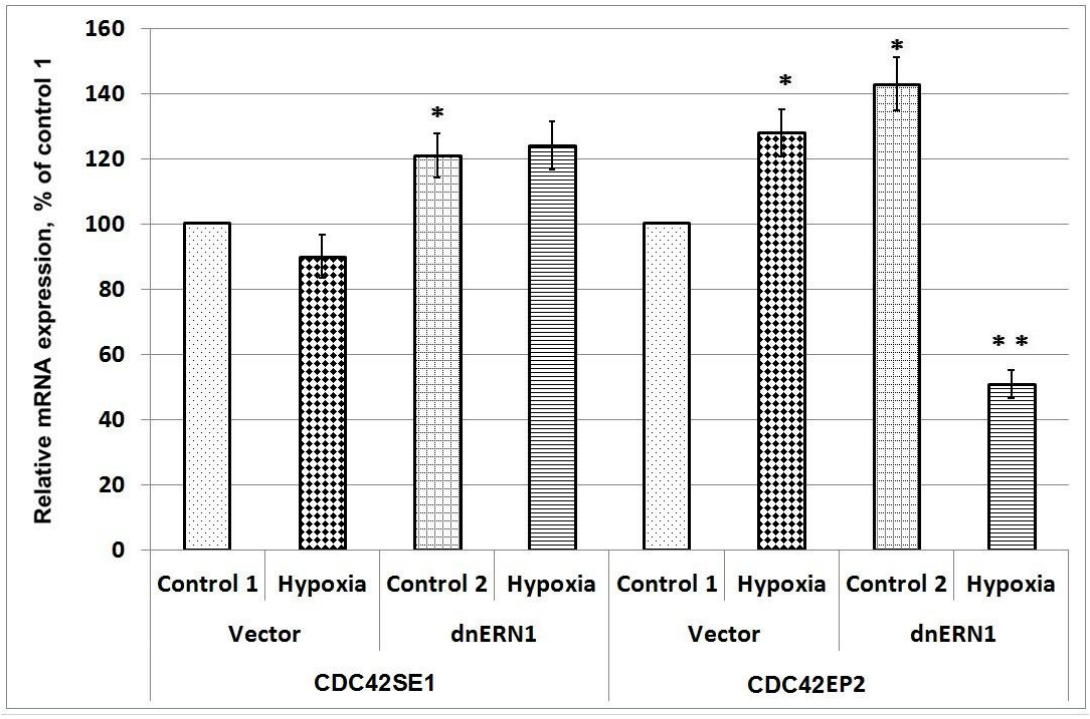

Fig. 2. Effect of hypoxia on the expression level of CDC42SE1 and CDC42EP2 in U87 glioma cells, transfected with vector pcDNA3.1 (Control) and cells, stable transfected with dominant/negative constructs of ERN1 signaling enzyme (dnERN1) into vector pcDNA3.1, measured by quantitative real-time PCR. *The level of these mRNA expressions was normalized to the expression of beta-actin. The changes in the expression of CDC42SE1 and CDC42EP2 mRNA in cells, transfected with dnERN1, were compared with control (100 %); n = 4; * – P < 0,05 as compared to control 1, ** – P < 0,05 as compared with control 2.*

This results clearly demonstrated that the expression of CDC20, CDC42SE1, CDC42EP2, CDC42EP3, CDC42EP4 and PAK4 genes in U87 glioma cells is dependent from ERN1 signaling enzyme function because blockade of this signaling enzyme changes the expression level all of these genes. Suppression of CDC20 and PAK4 as well as induction of CDC42SE1, CDC42EP2 and CDC42EP3 gene expressions in glioma cells with ERN1 signaling enzyme loss of function correlates with its decreased proliferation rate and possibly contributes in this effect, because CDC20 and PAK4 have pro-proliferative as well as cell survival functions and CDC42SE1, CDC42EP2 and CDC42EP3 negatively regulate the function of CDC42 (Drogat et al., 2007; Auf et al., 2010; Zhu et al., 2013; Xue et al., 2006; Wang et al., 2012; Zhang et al., 2012; Zhang et al., 2002).

Moreover, we have shown that hypoxia does not affect the expression of CDC42 as well as CDC42-effectors CDC42SE1 and CDC42EP3 mRNA expressions in cells with functional ERN1 (control glioma cells). At the same time, hypoxia increases the expression level of PAK4 and CDC42EP2 mRNA and decreases CDC20 mRNA in control glioma cells. However, the hypoxic regulation of the expression CDC42 as well as some CDC42-effectors genes mostly depends from the function of ERN1 signaling enzyme (Fig. 1 – 4). Thus, the blockade of ERN1 enzyme function leads to significant increase of the expression of CDC42 mRNA and strong decrease of CDC42EP2 gene expressions by hypoxia. Moreover, the suppression of signaling enzyme ERN1 enhances the effect of hypoxia on the expression of PAK4 mRNA but decrease its effect on CDC20 mRNA.

Thus, results of this study clearly demonstrated that the expression levels of most tested genes encoded CDC42 and its effectors as well as its hypoxic regulation is dependent from ERN1 signaling enzyme function and possibly participate in cell adaptive response to endoplasmic reticulum stress associated with hypoxia.

Conclusions. 1. It was shown that suppression of both enzymatic functions of sensor and signaling enzyme ERN1, the major component of endoplasmic reticulum stress signaling, increases the expression level of genes encoding of cell division cycle-20 (CDC20) as well as CDC42-effectors CDC42EP4 and PAK4 in glioma cell line U87. However, the expression level of CDC42 mRNA did not change significantly in glioma cells with ERN1 loss of function.

2. The blockade of ERN1 leads to increase the expression level of CDC42EP2, CDC42EP3 and CDC42SE1 mRNA, being more significant for CDC42EP3 gene.

3. Hypoxia does not affect the expression of CDC42 as well as CDC42-effectors CDC42SE1 and CDC42EP3 mRNA expressions, but increases PAK4 and CDC42EP2 and decreases CDC20 genes in cells with functional ERN1 (control glioma cells).

4. Hypoxic regulation of CDC42 as well as some CDC42-effectors gene expressions significantly depends from ERN1 signaling enzyme function because its blockade leads to increase of CDC42 and decrease of CDC42EP2 gene expressions by hypoxia.

5. Results of this study clearly demonstrated that the expression levels of genes encoded CDC42 and its effectors is mostly depend from ERN1 signaling enzyme function and possibly participate in cell adaptive response to endoplasmic reticulum stress associated with hypoxia.

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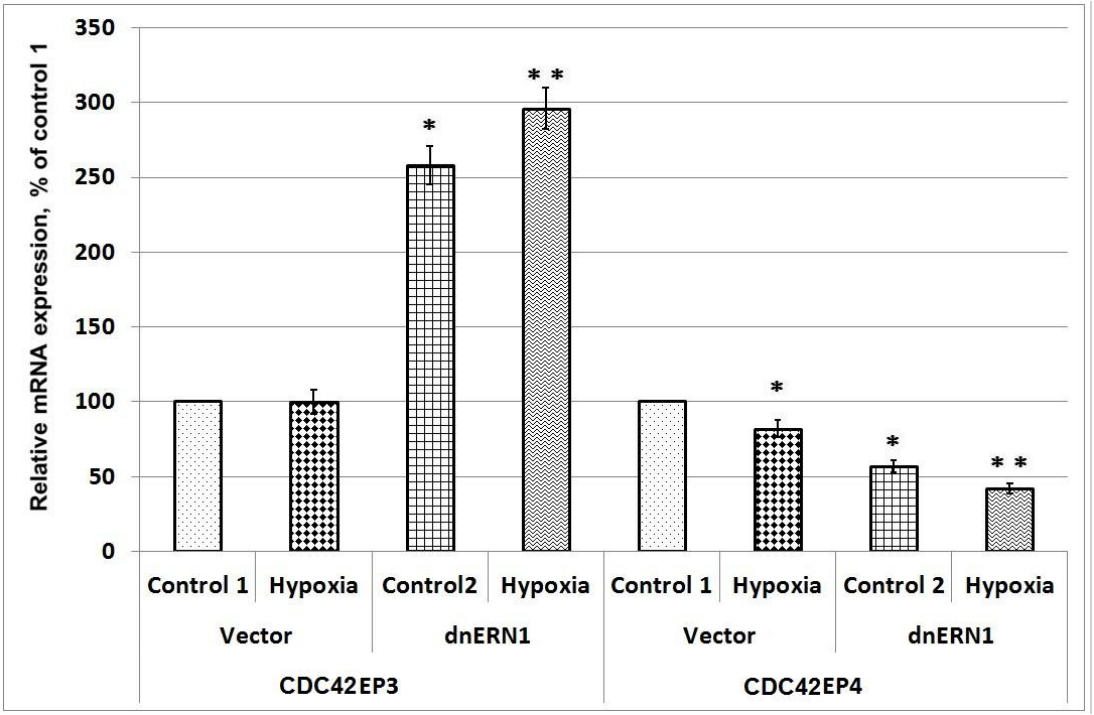

Fig. 3. Effect of hypoxia on the expression level of CDC42EP3 and CDC42EP4 in U87 glioma cells, transfected with vector pcDNA3.1 (Control) and cells, stable transfected with dominant/negative constructs of ERN1 signaling enzyme (dnERN1) into vector pcDNA3.1, measured by quantitative real-time PCR. *The level of these mRNA expressions was normalized to the expression of beta-actin. The changes in the expression of CDC42EP3 and CDC42EP4 mRNA in cells, transfected with dnERN1, were compared with control (100 %); n = 4; * – P < 0,05 as compared to control 1, ** – P < 0,05 as compared with control 2.*

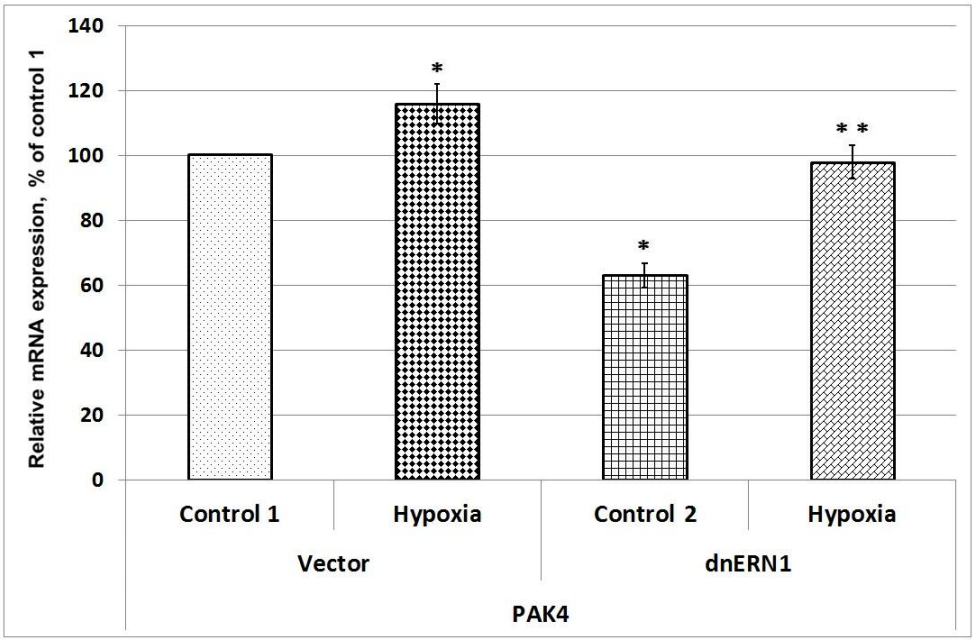

Fig. 4. Effect of hypoxia on the expression level of p21-activating kinase 4 (PAK4) in U87 glioma cells, transfected with vector pcDNA3.1 (Control) and cells, stable transfected with dominant/negative constructs of ERN1 signaling enzyme (dnERN1) into vector pcDNA3.1, measured by quantitative real-time PCR. *The level of these mRNA expressions was normalized to the expression of beta-actin. The changes in the expression of PAK4 mRNA in cells, transfected with dnERN1, were compared with control (100 %); n = 4; * – P < 0,05 as compared to control 1, ** – P < 0,05 as compared with control 2.*

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ЕКСПРЕСІЯ CDC20, CDC42 ТА CDC42-ЕФЕКТОРНИХ ГЕНІВ У КЛІТИНАХ ГЛІОМИ ЛІНІЇ U87 З ПРИГНІЧЕНОЮ ФУНКЦІЄЮ СИГНАЛЬНОГО ЕНЗИМУ ERN1: ВПЛИВ ГІПОКСІЇ

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Реакція на не згорнуті протеїни являє собою комплекс складних внутрішньоклітинних подій, що опосередковується переважно сигнальним ензимом ERN1 (від ендоплазматичного ретикулуму до ядра), виражено активується у клітинах злоякісних пухлин, зокрема через гіпоксію, і є необхідною для активації процесів неоваскуляризації та проліферації. Велика кількість протеїнів, що контролюють клітинний цикл, в тому числі цикліни та залежні від них кінази і їх інгібітори, є компонентами системи стресу ендоплазматичного ретикулуму і приймають участь у контролі клітинного циклу та процесів проліферації. Ми вивчали експресію генів, що задіяні в регуляції клітинного циклу і процесів проліферації у клітинах гліоми лінії U87 та її сублінії без ERN1 за умов гіпоксії. Встановлено, що блокада ERN1 приводить до зниження експресії мРНК CDC20 та ефекторів CDC42 (CDC42EP4 і PAK4), але при цьому збільшується рівень експресії мРНК CDC42EP2, CDC42EP3 і CDC42SE1. Разом з тим, рівень експресії мРНК CDC42 істотно не змінюється у клітинах гліоми з виключеною функцією ERN1. Більше того, нами показано, що гіпоксія не впливає на експресію мРНК як CDC42, так і ефекторів CDC42 (CDC42SE1 та CDC42EP3), але збільшує PAK4 та CDC42EP2 і знижує CDC20 у клітинах з функціональним ERN1 (контрольні клітини гліоми). Разом з тим, гіпоксична регуляція експресії генів CDC42 та деяких ефекторів CDC42 суттєво залежала від функції сигнального ензиму ERN1, оскільки його блокада призводила до збільшення експресії генів CDC42 та зниження CDC42EP2 за гіпоксії. Таким чином, результати дослідження свідчать про те, що рівень експресії більшості досліджених генів, які кодують CDC42 та його ефектори залежить від функції сигнального ензиму ERN1 і можливо приймає участь у адаптивній реакції клітин на стрес ендоплазматичного ретикулуму, асоційованого з гіпоксією.

Ключові слова: експресія мРНК, CDC20, CDC42, CDC42EP2, CDC42EP3, CDC42EP4, PAK4, виключення функції ERN1, клітини гліоми, гіпоксія.

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