SELENOM-KNOCKDOWN ENHANCES THE PROTECTIVE EFFECT OF A-172 CANCER CELLS AGAINST MSA-INDUCED ER-STRESS AND STAUROSPORINE-INDUCED APOPTOSIS

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Abstract. SELENOM is a highly conserved protein, presented in different species and classes of animals, belongs to the family of thioredoxin-like folding proteins. It is known that SELENOM is more expressed in the brain and is very sensitive to selenium deficiency in this organ; it is involved in the regulation of calcium signaling, redox homeostasis, and apoptosis in brain cells. This study showed that SELENOM-knockdown in human glioblastoma cells (A-172 cell line) contributed to a decrease in the number of apoptotic A-172 cells after 24 or 48 h treatment with the known apoptosis inducer staurosporine, as well as a significant decrease in the number of necrotic cells after 48 h treatment with this inductor. A decrease in SELENOM activity also led to a decrease in the Ca^{2+} ER-buffer capacity and influenced the level of mRNA expression of key ER-stress markers in glioblastoma cells.

Keywords: selenium, selenoprotein M, glioblastoma, apoptosis, ER-stress.

List of Abbreviations

ATF-4 – activating transcription factor-4
ATF-6 – activating transcription factor-6
ER – endoplasmic reticulum
IRE1 – α-isoform of inositol-dependent enzyme 1 type
KD – knockdown
MSA – methylseleninic acid
PKR – protein kinase R-like
PERK – PKR–like ER-kinase
SELENOM – selenoprotein M
ST – Staurosporine
UPR – unfolded protein response
XBPs – X-box binding protein spliced
XBPu – X-box binding protein unspliced

Introduction

SELENOM is a selenoprotein localized in the endoplasmic reticulum (ER), which was identified using bioinformatic approaches (Korotkov *et al.*, 2002; Zhou *et al.*, 2011). SELENOM is a highly conserved protein found in different species and classes of animals and belongs to the family of proteins with a thioredoxin-like folding: it has a conservative CXXU motif in the catalytic center (where C is cysteine, X is any two amino acids, U is selenocysteine) (Dikiy *et al.*, 2007). Human SELENOM is expressed in many tissues, but to a greater extent in the brain (Zhou et al., 2011), is most sensitive to selenium (Se) deficiency in this organ and can serve as a molecular biomarker of Se status, and SELENOM is also involved in the regulation of human neurogenerative diseases (Huang *et al.*, 2016). Similar results were shown in HT22 hippocampal cells and C8–D1A cerebellar cells: stable overexpression of SELENOM prevented oxidative cell damage caused by hydrogen peroxide (Reeves *et al.*, 2010). On the contrary, SELENOM-KD in rats led to an increase in glutathione peroxidase and thioredoxin reductase activities in the brain, liver, lungs and, to a lesser extent, in the kidneys and heart, while an increase in superoxide dismutase activity was observed only in the brain. Thus, SELENOM in different tissues can regulate the activity of two antioxidant enzymes in different ways. In addition, overexpression of SELENOM in HT22 and C8–D1A cells increased the concentration of cytosolic calcium in response to oxidative stress and, possibly, participated in the regulation of

apoptosis by blocking or delaying it (Hwang *et al*., 2008).

In addition, we have repeatedly demonstrated the important role of SELENOM in the regulation of ER–stress caused by various inducers of both Se and non-Se nature (Goltyaev et al., 2020; Kuznetsova *et al.*, 2018; Turovsky & Varlamova, 2021; Varlamova, 2020; Varlamova *et al.*, 2021). A search was carried out for physiological partners of SELENOM in various human cancer cells, as a result of which two isoforms of cytoplasmic actin were identified as possible physiological partners of this selenoprotein: cytoplasmic actin 1 (cytoskeletal β-actin) and cytoplasmic actin 2 (cytoskeletal γ-actin) (Varlamova *et al.*, 2019). Considering that SELENOM is a homologue of SELENOF-another ER-resident selenoprotein, which is involved in membrane blebbing, the functional relationship with actin, a key cytoskeleton protein, may also indicate the participation of SELENOM in the membrane blebbing that occurs at the late stages of apoptosis. Studies of the role of SELENOM in carcinogenesis are also limited to a small number of studies (Guerriero et al., 2014; Varlamova *et al.*, 2018).

This study showed that SELENOM-knockdown (SELENOM-KD) in human glioblastoma cells (A-172 cell line) contributed to a decrease in the number of apoptotic A-172 cells after 24 or 48 h treatment with the known apoptosis inducer staurosporine, as well as a significant decrease in the number of necrotic cells after 48 h treatment with this inductor. A decrease in SELENOM activity also led to a decrease in the Ca^{2+} ER-buffer capacity and influenced the level of mRNA expression of key ER-stress markers in glioblastoma cells. In this work, we used a Se-containing inducer of ER– stress, methylseleninic acid (MSA), since we previously selected the concentrations of this inductor that cause adaptive and prolonged ER-stress using the example of various cancer cells (Goltyaev *et al.*, 2020; Varlamova *et al.*, 2019; Varlamova *et al.*, 2021).

Materials and Methods

Cell culture. Cell line A-172 were purchased from ATCC (Manassas, VA, USA) and were grown on round coverslips for 48 h in a CO_2-incubator in DMEM medium supplemented with 10% fetal bovine serum until a confluence of 80–95% was achieved. Cell cultures were used from the third passage.

SELENOM-KD by RNA interference and transduction with lentiviral particles. Gene KD by RNA interference was performed using a vector containing a sequence of shRNAs, which were independently designed using the online resource https://www.invivogen.com/sirna-wizard. The construction of lentiviral vectors and the production of lentiviral particles were carried out at the Evrogen (Russia). Treatment of cancer cells with the preparation of lentiviral particles was carried out in 24-well plates at the stage of logarithmic cell growth with a confluence of 60–70%. After 24 h from the moment of transduction, the complete DMEM medium was changed, and after 72 h the result of the efficiency of transduction was assessed by fluorescence microscopy. Selection of transduced cells, every 4 days, was by adding puromycin (1–3 μg/ml medium) to the culture medium. Evaluation of the efficiency of KD after transduction with lentiviral particles was by real-time PCR and Western blotting.

Cell staining. The concentration of calcium ions in the cytoplasm ($[Ca^{2+}]i$) was assessed using a two-wave probe Fura-2 in accordance with the well-known method (Grynkiewicz *et al.*, 1985). For the staining of adipocytes, Fura-2 AM ester was used at a final concentration of 4 μM in Hanks solution, containing (in mM): 156 NaCl, 3 KCl, 1 $MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose and 10 HEPES, pH 7.4. 200 μL of a freshly prepared dye solution was added to each coverslip with cell culture and incubated in a thermostat for 40 min at 37 °C. After that, the culture was washed with Hanks solution and incubated for 10–15 min to complete the deesterification of the dye.

Fluorescence measurements. The measurement of cytosolic $[Ca^{2+}]$ was performed by fluorescent microscopy using Fura-2AM (Molecular probes, USA), a ratiometric fluo-

rescent calcium indicator. Cells were loaded with the probe dissolved in Hanks balanced salt solution (HBSS), containing 10 mM HEPES, pH 7.4, at a final concentration of 5 μM at 370 °C for 40 min with subsequent 15 min washout. The coverslip containing the cells loaded with Fura-2 was then mounted in the experimental chamber. During the experiment we used a perfusion system, which enables complete replacement of the cell bathing solution within 30 seconds. We used an Axiovert 200M based imaging system (Carl Zeiss, Germany) equipped with HBO100 mercury lamp, AxioCam HSm CCD camera and MAC5000 high speed excitation filter wheel. Fura-2 fluorescence was excited at two wavelengths using band-pass filters BP 340/30 and BP 387/15; fluorescence was registered in the wavelength range of 465–555 nm. Excitation light intensity was lowered using 25 and 5% neutral density filters in order to prevent phototoxicity. Image frames were acquired at 3 seconds intervals with a Plan Neofluar 10×/0.3 objective. The time lapse image sequences were analyzed using ImageJ 1.44 (NIH Image, Bethesda, MD). Graphs were plotted using OriginPro 8.0 software Microcal Software Inc., Northampton, MA). Statistical analysis was performed using the same software. Results are presented as means ± standard error (SE) or as representative calcium signal of the cells.

Assessment of cell viability and apoptosis. Cell death (apoptosis or necrosis processes) in the cell culture was assessed by simultaneous staining of cells with Propidium iodide (PI, 1 μM) and Hoechst 33342 (HO342, 1 μM). Viable cells are not permeable to PI, while Hoechst 33342 penetrates through the plasma membrane and staining the chromatin. According to a commonly used method (Gaidin et al., 2019), A-172 cells were defined as apoptotic if the intensity of Hoechst 33342 fluorescence was 3–4 times higher compared to Hoechst 33342 fluorescence in healthy cells, indicating chromatin condensation, which can occur as a result of apoptosis induction. The fluorescence of the probes was registered with a fluorescent system based on an inverted fluorescent microscope Axio Observer Z1 equipped with a high-speed monochrome CCD-camera Hamamatsu ORCA-Flash 2.8. The Lambda DG-4 Plus illuminator (Sutter Instruments, USA) was used as a source of excitation. To excite and record fluorescence of the probes we used: Filter Set 01 with excitation filter BP 365/12, beam splitter FT395, emission filter LP 397; Filter Set 20 with excitation filter BP 546/12, beam splitter FT560, emission filter BP 575–640. We used objective HCX PL APO 20.0x0.70 IMM UV, refraction index 1.52. Camera settings is 500 pixels X 500 pixels (Voxel Size 0.724 μm X 0.723 μm), binning 2 X 2, resolution 14 bits. Five different fields of view were analyzed for each coverslip with cells. Each experiment was repeated three times with separate cell cultures.

Extraction of RNA and Real-time polymerase chain reaction (*RT–qPCR*). Total RNA from A-172 cells after 24 h of treatment with various concentrations of SeNP was isolated using ExtractRNA reagent according to the manufacturer's instructions (Evrogene, Russia). The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First–strand cDNA was synthesized from 1–2 μg of total RNA using MMLV reverse transcriptase according to the manufacturer's instructions (Evrogene, Russia). Real-time qPCRs were performed in a 25μL reaction mixture containing SYBR Green I PCR Master Mix (Evrogene, Russia) and 300 nM of the appropriate primers (Table 1). The PCR procedure consisted of 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization, and results were expressed as $2^{-\Delta(\Delta Ct)}$.

Statistical analysis. All presented data were obtained from at least three cell cultures from 2–3 different passages. All values are given as mean ± standard error (SE) or as individual Ca^{2+}-responses.

Table 1

Primers for Real-time PCR

Gene name	Forward primer 5'–>3'	Reverse primer 5'–>3'
GAPDH	ACATCGCTCAGACACCATG	GCCAGTGAGCTTCCCGTT
SELENOM	AGCCTCCTGTTGCCTCCGC	AGGTCAGCGTGGTCCGAAG
ATF–4	GTGTTCTCTGTGGGTCTGCC	GACCCTTTTCTTCCCCCTTG
ATF–6	AACCCTAGTGTGAGCCCTGC	GTTCAGAGCACCCTGAAGA
XBPu	ACTCAGACTACGTGCACCTC	GTCAATACCGCCAGAATCC
XBPs	CTGAGTCCGCAGCGGTGCAGG	GGTCCAAGTTGTCCAGAATG

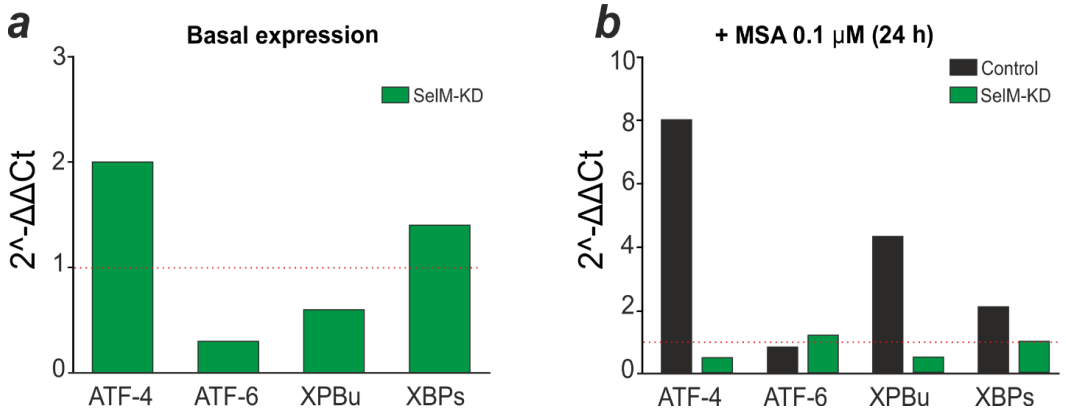

Fig. 1. The effect of SELENOM-KD on the baseline (a) and the level of expression of genes encoding the ER-stress marker proteins induced by the application of 0.1 μM MSA (b). Dash line – expression level in A-172 cells without SELENOM-KD (a) and without MSA application (b)

Results

SELENOM-KD affects mRNA expression of some key ER-stress markers. In order to test whether SELENOM-KD affects the expression of mRNA of key marker genes of the three ER-stress signaling pathways, a series of experiments on Real-time PCR was performed.

Unfolded protein response (UPR) is a complex of closely interconnected signaling branches, which is represented by a triad of transmembrane proteins-PERK (PKR-like ER-kinase), IRE1 (α-isoform of inositol-dependent enzyme 1 type) and ATF6 (activating transcription factor 6). It is known that upon activation of the PERK signaling pathway, selective translation of transcription factors, in particular ATF-4, is observed (Harding *et al.*, 2000; Vallejo *et al.*, 1993). XBP1 is a transcription factor whose dynamic form is controlled by alternative splicing under ER-stress: IRE1α removes the 26–nucleotide intron, which leads to a frame shift and translation of the biologically active form of XBP1–XBP1s (Calfon *et al.*, 2002). ATF-6 is a type II transmembrane protein that leaves the ER membrane under ER-stress and is sent to the Golgi apparatus, where it is processed by proteases and acquires an active form (Okada *et al.*, 2002).

According to the results shown in Fig. 1a, it can be concluded that SELENOM-KD promotes an increase in the expression of ATF-4 mRNA, a decrease in the expression of ATF-6 mRNA by almost half, and practically does not affect the expression of XBP mRNA. Whereas under the ER–stress, caused by incubating cells with 0.1 μM MSA for 24 h, SELENOM-KD, on the contrary, promoted a sharp decrease in the ATF-4 mRNA expression, as well as the unspliced form of XBP, a slight increase in the expression level of spliced mRNA of XBP, and practically did not change the expression of ATF-6 (Fig. 1b).

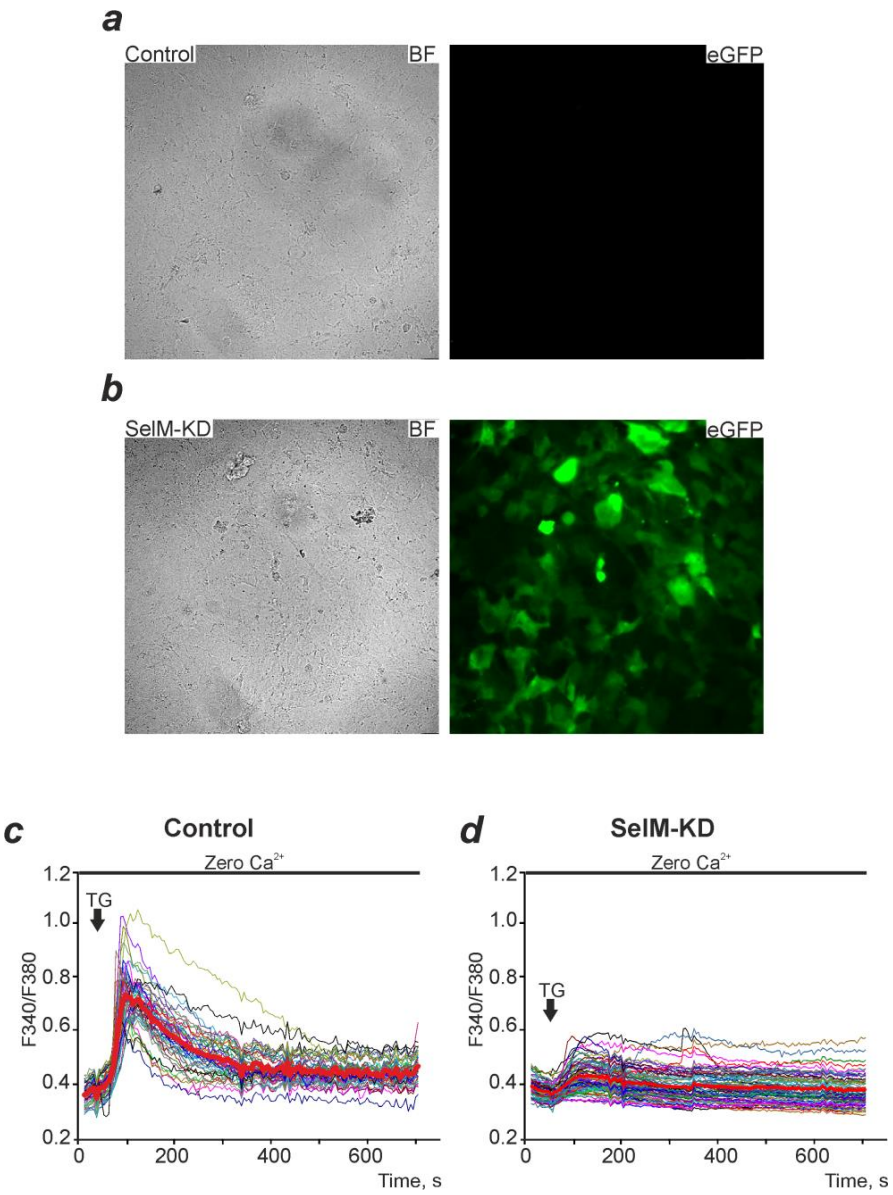

Fig. 2. SELENOM–KD reduces the Ca^{2+}–buffer capacity of ER
a, b – Control (a) and SELENOM–KD (b) A–172 cells in transmitted light (BF) and eGFP fluorescence (reflects the efficiency SELENOM–KD); c, d – Ca^{2+}–signals of control (c) and SELENOM–KD (d) A–172 cells to the application of tapsigargin (TG, 10 μM) in a calcium–free medium. Shown are individual cell responses and their mean (red curves)

SELENOM-KD reduces the Ca^{2+}-buffer capacity of ER. Figures 2a and c show that SELENOM-KD has no effect on the proliferation rate of A-172 cells, since the cells reach confluence at a rate characteristic of control cells. To measure the Ca^{2+} capacity of the ER, the cells were loaded with a Ca^{2+}-sensitive probe Fura-2 and the SERCA blocker, thapsigargin, was added in a calcium-free medium when the influx of Ca^{2+} ions from outside was completely absent (Fig. 2c, d). In control cells (Fig. 2c), thapsigargin induces Ca^{2+} signals, on average, 2 times higher in amplitude compared to cells with SELENOM-KD (Fig. 2d).

SELENOM-KD leads to a disruption in the functioning of the ER in the A-172 cells, which is expressed in a decrease (the ER is almost completely empty) of its Ca^{2+} capacity; this

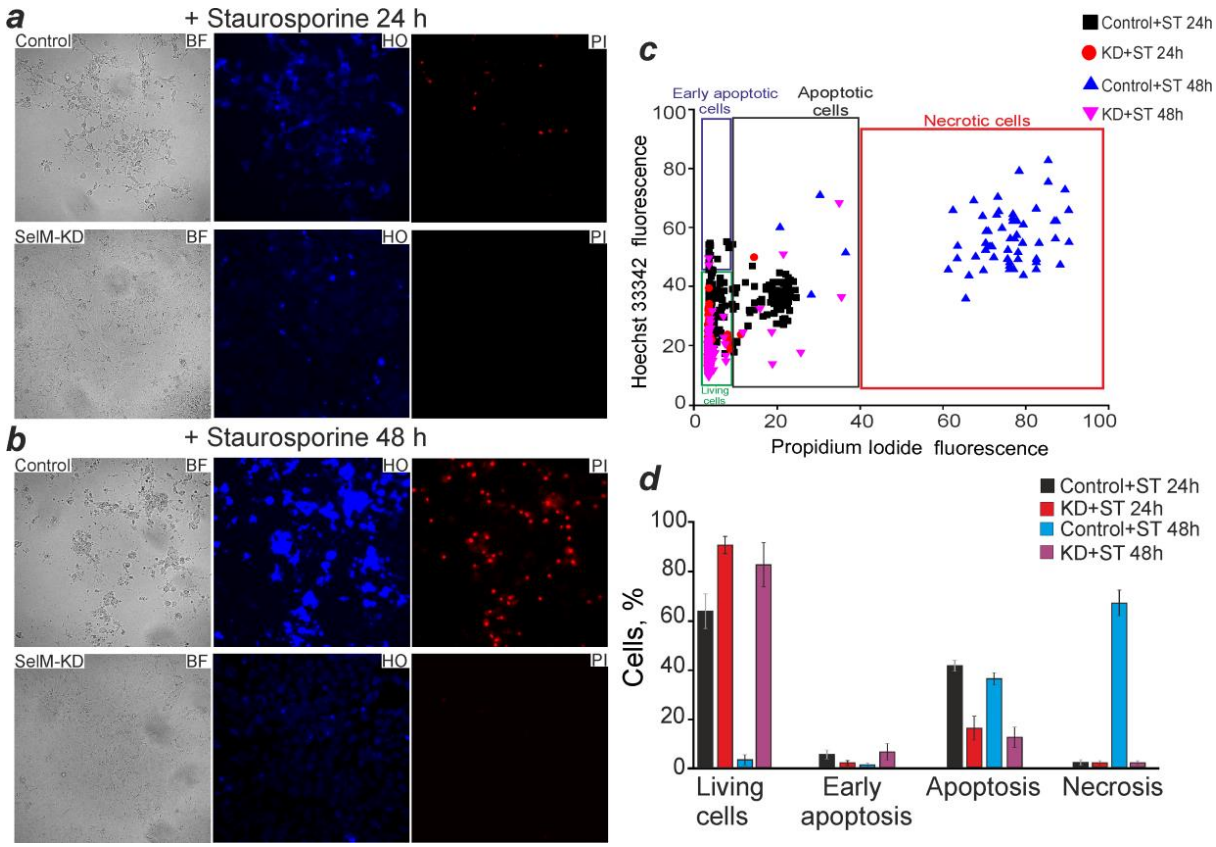

Fig. 3. The effect of SELENOM-KD on the viability of A-172 cells under apoptosis induced by the ST (5 μM).
a, b – Control and SELENOM-KD A-172 cells in transmitted light (BF), nuclei stained with Hoechst 33342 (HO, blue) and Propidium iodide (PI, red, necrotic cells) fluorescent probes after 24 h (a) and 48 h (b) incubation with ST;
c – Cytogram demonstrating the viability of control and SELENOM–KD A-172 cells after 24 h and 48 h incubation with ST;
d – Effect of SELENOM-KD on cell viability, activation of apoptosis and necrosis 24 h and 48 h after ST application. X-axis-the intensity of PI fluorescence; Y-axis-the intensity of Hoechst 33342 fluorescence

protein may probably be associated with the mechanisms of ER pumping and emptying.

SELENOM-KD provided protective anti-apoptotic effect on glioblastoma cells when exposed to Staurosporine. Staurosporine (ST) is a well-known inducer of apoptosis, the molecular mechanisms of which are poorly understood. According to the results shown in Fig. 3 (a-d), it can be concluded that treatment of cells with ST for 24 h and 48 h led to a decrease in the viability of A-172 cells by approximately 40% and 90%, respectively. Whereas under conditions of SELENOM-KD, a significant protective effect from ST was observed. The number of cells without apoptosis increased and amounted to about 90% both at 24 h and at 48 h treatment with ST.

Treatment of A-172 cells with ST for 24 h and 48 h both under SELENOM-KD conditions and without it practically did not lead to the development of early apoptosis in cells. However, the number of cells in the late stages of apoptosis increased by approximately 40% at 24 h or 48 h exposure to ST. SELENOM-KD led to a decrease in the number of cells with apoptosis caused by 24 h and 48 h treatment with ST, almost two–fold compared to the samples without KD. Interestingly, only 48 h (but not 24 h) treatment of cells with ST led to a significant growth of cells with necrosis (by more than

60%), while SELENOM–KD almost completely suppressed this effect, reducing the number of necrotic cells to 1–5%.

Discussion

SELENOM is one of the 25 mammalian selenoproteins, represented also in other classes of animals, which indicates its evolutionary conservatism. In addition, SELENOM is also an ER-resident selenoprotein and, along with SELENOF and SELENOT, belongs to the family of proteins with a thioredoxin-like folding (Dikiy *et al.*, 2007). Earlier, we and other authors have repeatedly shown the active role of this selenoprotein in the regulation of ER-stress caused by various sources of Se and non-Se nature (Goltyaev *et al.*, 2020; Kuznetsova *et al.*, 2018; Turovsky & Varlamova, 2021; Varlamova, 2020; Varlamova *et al.*, 2021). It was found that the expression of its mRNA and the activity of SELENOM significantly depend on the nature and concentration of the selected ER-stress inducer; however, the functions of this selenoprotein in the regulation of ER-stress and carcinogenesis are still unclear. Therefore, the aim of this work was to study the consequences of SELENOM-KD: a change in the expression of key markers of three UPR-signaling pathways, a change in the concentration of calcium ions in the ER, and a change in cell viability.

It is known that SELENOM is largely localized in the brain, especially in the cells of the hippocampus and cerebellum; therefore, the study of M functions in the cells of this organ is highly relevant (Huang *et al.*, 2016; Reeves *et al.*, 2010; Zhou *et al.*, 2011). In this regard, in our work, we performed a SELENOM-KD in human brain cancer cells A-172 (glioblastoma).

Since the main goal of this work was to study the role of SELENOM in the regulation of ER–stress, we carried out a series of experiments to study the effect of SELENOM-KD in glioblastoma cells under ER-stress induced by MSA, a well-known ER-stress inducer of Se nature, on the expression levels of key markers of three signaling pathways UPR (ATF-4, XBP and ATF-6). It was found, that SELENOM-KD promotes an increase in the expression of ATF-4 mRNA, a decrease in the expression of ATF-6 mRNA by almost half, and practically does not affect the expression of XBP mRNA. Whereas under the ER-stress, caused by incubating cells with 0.1 μM MSA for 24 h, SELENOM-KD, on the contrary, promoted a sharp decrease in the ATF-4 mRNA expression, as well as the unspliced form of XBP, a slight increase in the expression level of spliced mRNA of XBP, and practically did not change the expression of ATF-6 (Fig. 1).

It is known that ATF-4, transcription factor and a key participant of the PERK pathway of the UPR, has the ability to induce antioxidant defense genes. This transcription factor reacts to changes in the ratio of reduced and oxidized SH-groups in proteins and has a wide action potential, activating promoters of antioxidants, detoxification enzymes, chaperones, and mediators of cell proliferation and survival (Cullinan S.B. *et al.*, 2003). PERK activity is important not only at the cellular, but also at the tissue level; in particular, it is known that PERK inhibits proliferation by suspending the cell cycle in the G1 phase under conditions of pronounced hypoxia (Brewer & Diehl, 2000).

Thus, in the absence of ER–stress, SELENOM-KD tends to protect A-172 cells from oxidative stress caused by an increase in the expression of ATF-4 mRNA. However, under the conditions of ER stress induced by the action of MSA, on the contrary, it suppresses the expression of this transcription factor. Therefore, SELENOM can perform the protective antioxidant function of human glioblastoma cells under ER–stress caused by MSA.

We found that SELENOM-KD has no effect on the proliferation rate of A-172 cells, since the cells reach confluence at a rate characteristic of control cells (Fig. 2a, b). In addition, it was shown that SELENOM-KD has a pronounced protective effect of human glioblastoma cells from apoptotic and necrotic death caused by the action of ST.

It is known that Ca^{2+} in the ER perform a complex of physiological functions, and the level of these ions in the ER regulates metabolic signaling pathways in non-excitable cells (Turovsky *et al.*, 2021). While an increase in $[Ca^{2+}]i$ is responsible for the induction of necro-

sis and apoptosis in glial and nerve cells (Turovsky *et al.*, 2021; Gaidin *et al.*, 2019). Therefore, it was highly relevant to study the role of SELENOM-KD on the regulation of Ca^{2+} levels in the ER. It was shown that SELENOM-KD leads to a disruption in the functioning of the ER in the A-172 cells, which is expressed in a decrease (the ER is almost completely empty) of its Ca^{2+} capacity; this protein may probably be associated with the mechanisms of ER pumping and emptying (Fig. 2c).

We have previously shown that the mobilization of Ca^{2+} ions from the ER via IP3R led to the induction of apoptosis in A-172 cells when Se nanoparticles were added to them (Turovsky & Varlamova, 2021), which may be due to the action on selenoproteins, including SELENOM. However, in this work SELENOM–KD inactivated of the signaling pathway leading to apoptosis of A-172 cells, and this protein can be considered in the future as an important target for anticancer therapy.

In addition, it was shown that SELENOM-KD promotes an increase in the number of A-172 cells, which have no signs of necrosis and apoptosis, by approximately 30–80%, depending on the time of exposure cells with ST (Fig. 3). These results indicate the important role of SELENOM in suppressing the viability of human glioblastoma cells, stimulating apoptosis or necrosis, when exposed to them by an apoptosis inducer, in particular ST.

Conclusion

SELENOM-KD has a pronounced protective effect of human glioblastoma cells from apoptotic and necrotic death caused by the action of ST and leads to a disruption in the functioning of the ER in the A-172 cells, which is expressed in a decrease of its Ca^{2+} capacity. In the absence of ER-stress, SELENOM-KD tends to protect A-172 cells from oxidative stress caused by an increase in the expression of ATF-4 mRNA. SELENOM can be considered in the future as an important target for anticancer therapy.

Acknowledgements

This study was conducted in the framework of the State assignment of PSCBR RAS, Project 0191-2019-0019.

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