

SELENOT–Knockdown Leads to Reducing the Expression of Key Enzymes of ERAD–System in Cancer Cells Under ER–Stress

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Abstract

SELENOT is one of the seven selenoproteins localized in the ER. The purpose of this work was to study the effect of SELENOT-knockdown on mRNA expression of a large number of genes (ER-stress markers, physiological partners of SELENOT, ER-resident selenoproteins, other selenoproteins) under conditions of ER-stress caused by both selenium-containing compounds (sodium selenite and methylseleninic acid) and non-selenium-containing compounds (dithiothriitol) by the example of two human cancer cell lines A-172 (human glioblastoma) and Caco-2 (human colorectal adenocarcinoma). In the course of this work, it was found that SELENOT-knockdown does not significantly affect viability and proliferative properties, redox homeostasis in A-172 and Caco-2 cells and the acquisition of signs of normal cells by them, does not significantly affect the expression of ER-stress marker genes, ER-resident selenoproteins, with the exception of DIO2 and SELENOM. But it has a significant effect on reducing the levels of mRNA expression of AMFR and RNF5 are important enzymes of the ERAD-system, disrupting its work and leading to the accumulation of proteins with incorrect folding, thereby only aggravating ER stress.

1. Introduction

Homo sapiens gene of selenoprotein SELENOT (*hSELENOT*) is located on chromosome 3 and is represented by six exons, with the TGA-selenocystein codon in the second exon. The nucleotide sequence of SELENOT is highly conserved: 97–100% homology between the hSELENOT sequence and other mammalian species, 95% with the chicken sequence and 89% with the frog sequence. In addition, SELENOT is one of the oldest vertebrate selenoproteins, since cysteine homologues of this protein are found in protozoa, terrestrial and aquatic plants, and insects [1,2]. Selenocysteine (U) is located in the 49th position of the N-terminal region of hSELENOT in the CVSU motif, which is the redox center of the protein. The arrangement of the secondary structure elements of SELENOT and homology with thioredoxin allows it to be attributed to the family of proteins with a thioredoxin-like fold, in which the CVSU motif is located between the β 1-structure and the α 1-helix. hSELENOT (22.3 kDa) has a signal peptide represented by 19 amino acid residues, as well as a highly hydrophobic region of 16 amino acid residues in position 87–102 aa, which is a transmembrane domain [1,3,4]. Shchedrina et al. suggested the presence of two hydrophobic domains within the 87–102 amino acid residues, which could allow SELENOT to double-cross the ER membrane [5]. Alternatively, it is assumed that SELENOT is partially bound to the membrane [6]. The presence of thioredoxin-like folding in this protein suggests the presence of an enzymatic activity similar to thioredoxin-reductase, which was confirmed in studies of the ability of SELENOT to reduce 5,5'-dithio-bis (2-dinitrobenzoic acid) to 5-thio-2-nitrobenzoic acid. However, glutathione peroxidase activity was not detected in this protein [7].

Analysis of the SELENOT mRNA expression levels in different classes of organisms revealed its enhancement during embryonic ontogenesis and a significant postnatal decrease in its expression in various organs and tissues, with the exception of the pituitary gland, thyroid, pancreas, and testes [8,9]. The molecular mechanisms of such differential expression of SELENOT in A-172 and Caco-2 lines

unclear, however, it has been shown that its expression can be induced by regulatory signals under certain conditions. In neuroendocrine cells, the expression of SELENOT is regulated by the levels of cAMP and Ca^{2+} ions through the PACAP-peptide, which activates pituitary adenylate cyclase [3].

It has been shown that SELENOT interacts with keratinocyte-associated protein 2 (KRTCAP2, KCP2), which is a subunit of the oligosaccharyltransferase protein complex (OST complex) involved in N-glycosylation of proteins. In addition, SELENOT interacts with other subunits of the A-type OST-complex as a decrease in SELENOT expression leads to disruption of the N-glycosylation [9,10]. The molecular mechanisms of regulation of SELENOT processes of N-glycosylation are still not clear, but several studies indicate that the protein can specifically participate in the N-glycosylation of proteins with disulfide bridges, for example, glycoprotein hormones with disulfide bonds [8]. Using co-immunoprecipitation, it was shown that, under native conditions, SELENOT, in addition to KCP2, also binds to STT3A and OST 48, but not to the STT3B. Interestingly, with a decrease in the amount of SELENOT protein by almost 80% in AtT-20 cells, a decrease in the levels of proteins KCP2, STT3A, OST48, but not STT3B, was observed, while the level of mRNA of the genes encoding them did not change. In addition, when SELENOT was silenced in these cells, a sharp decrease in the expression of the GP78 (72%) and RNF5 (76%) genes was observed [11]. This may indicate that, with a decrease in SELENOT activity, proteins with improper folding may accumulate in cells due to dysfunction of the ERAD system.

Taking into account the information already available about SELENOT, the purpose of this work was to study the effect of SELENOT-knockdown on mRNA expression of a large number of genes (ER-stress markers, physiological partners of SELENOT, ER-resident selenoproteins, other selenoproteins) under conditions of ER-stress caused by both selenium-containing compounds (sodium selenite and methylseleninic acid) and non-selenium-containing compounds (dithiothreitol) using as a model the two human cancer cell lines A-172 (human glioblastoma) and Caco-2 (human colorectal adenocarcinoma).

2. Results

2.1. Silencing of SELENOT mRNA expression and protein activity most markedly affected the expression of DIO2, RNF5, GADD34, PUMA, BAX, GPX2, and GPX3 in A-172 and Caco-2 cancer cells.

By transduction of the studied cancer cell lines with lentiviral particles, it was possible to reduced the expression of the SELENOT mRNA by 10 times (by 90%) and the amount of protein by approximately 3-4 times (Fig.1-3). After that, the effect of SELENOT silencing on the mRNA expression of six other ER-resident selenoproteins, four selenium-containing glutathione peroxidases and three thioredoxin reductases, putative physiological partners of SELENOT in ER and key ER-stress markers were investigated in A-172 and Caco-2 cancer cells. The results of real-time PCR indicate that out of 36

genes studied by us, mRNA expression changed in only seven of them by more than two times in almost the same way in both cell lines.

Among the ER-resident selenoproteins, SELENOT-knockdown influenced only the mRNA expression of DIO2, which decreased in both cell lines by about 30% (Fig.1 and 2 a). However, the decrease in the level of mRNA was not associated with a decrease of DIO2 protein level, as revealed by Western blot analysis (Fig. 3). Surprisingly, the mRNA expression of neither SELENOM nor SELENOF practically changed, which, along with SELENOT, have a thioredoxin-like folding. Among the proteins, the expression of which in previous studies significantly changed with a decrease in SELENOT expression, in this work only the expression of RNF5 was reacted most actively. So the expression of its mRNA in A-172 cells decreased to 20%, while in Caco-2 cells the decrease was non-significant (Fig. 1 and 2 b). However, no changed protein levels could be detected by Western blot analysis (Fig. 3). SELENOT-silencing also influences the expression of some markers of ER-stress: GADD34, PUMA, BAX (Fig. 1 and 2 c). In both cell lines there was a trend to decrease expression levels of these genes. The expression of two of the four studied glutathione peroxidases in Caco-2 cells also changed. There was a sharp avoid by 10 times. This increase was only observed in Caco-2. In A-172 the increase was by about 80%. The mRNA expression of GPX2 and, conversely, an increase in GPX3 expression by more than 4 times (Fig. 1 and 2 d).

2.2. SELENOT-knockdown in A-172 and Caco-2 cancer cells significantly suppresses the expression of selenoprotein SELENOM mRNA under ER-stress conditions.

Previously, we selected the concentrations of SS, MSA and DTT, which, when treated with A-172 and Caco-2 cells, caused a significant increase in mRNA expression of SELENOT, but these data have not yet been published. Under ER-stress conditions, caused by the treatment of A-172 and Caco-2 cancer cells with 0.1 μ M MSA, 1 mM DTT or 5 mM DTT during 24 h, a sharp decrease in the expression of SELENOM mRNA was observed (Fig. 4 and 5 a). In A-172 cells, such a decrease was observed with prolonged ER-stress caused by both 1mM DTT or 5 mM DTT. In Caco-2 cells, a decrease in the expression of its mRNA was also observed when cells were treated with 0.1 μ M MSA. As Western blot analysis revealed, the decreased mRNA levels were associated with decreased protein levels (Fig. 6 and 7). Interestingly, in both cell lines, SELENOT-knockdown under ER-stress did not affect the expression of DIO2, as it was also observed in untreated control cells, without ER stress sources. Only a slight increase in mRNA expression was recorded in Caco-2 cells when treated with 0.1 μ M SS and 0.1 μ M MSA during 24 h. In addition, some increase in SOLENON mRNA expression was observed in Caco-2 cells after treatment with 1mM DTT or 5 mM DTT. (As good as nothing happens in A-172).

2.3. SELENOT–knockdown decreases the expression of its two putative physiological partners AMFR and RNF5

Among the nine previously identified physiological partners of the SELENOT, only two of them showed a decrease in mRNA expression in the cells of both cancer lines under conditions of SELENOT–knockdown and simultaneously ER–stress. In A–172 cells, a decrease in the activity of the SELENOT led to a decrease in the mRNA expression of AMFR under the conditions of ER–stress caused by 0.1 μ M MSA, 1 mM DTT and 5 mM DTT on average by 60–90% (Fig. 4 and 5 b). In Caco–2 cells, the expression of its mRNA decreased only when the cells were treated with 1 mM and 5 mM DTT (70–90%) (Fig.5 b). There was also a decrease in the mRNA expression of another partner of SELENOT, RNF5, in A–172 and Caco–2 cells, especially when the cells were treated with 1 mM DTT (by 60–80%). A tendency to decrease in the content of AMFR and RNF5 proteins in A–172 and Caco–2 cells, by analogy with data on mRNA expression of their genes was also established by Western blot analysis (Fig. 6 and 7).

2.4. SELENOT–knockdown decreases the mRNA expression of a number of ER–stress marker genes and some glutathione peroxidases and thioredoxin reductases under ER–stress

In A–172 cells, SELENOT–knockdown both under the ER–stress conditions induced by treatment with 1mM DTT, and without treatment to any sources of ER–stress, caused a decrease in the mRNA expression of GADD34, PUMA, BAX. When treated with 1 mM DTT, a decrease in the expression of mRNA of the CHOP and BAK genes was also observed (Fig.4 c). However, SELENOT–knockdown had no effect on the expression of ER–stress marker genes in Caco–2 cells (Fig.5 c).

In addition, the ambiguous character of changes in the expression of the genes of some glutathione peroxidases and thioredoxin reductases was observed after SELENOT–knockdown. When A–172 cells were treated with 0.1 μ M SS, an increase in the expression of GPX4 mRNA was observed more than twofold (Fig.4 d). However, the threefold increase in expression of TXNRD1 under these conditions should be mentioned, while in Caco–2 cells under ER–stress caused by the action of 5 mM DTT, a decrease in expression GPX4, TXNRD 1–3 was observed (Fig.5 d).

2.5. SELENOT–knockdown does not affect the viability and proliferation of A–172 and Caco–2 cells, does not contribute to their transformation into normal.

It is shown that SELENOT– knockdown did not attenuate their cancer characteristics (Fig. 8 a, b).

To test whether SELENOT knockdown leads to a partial or complete reversion of cancer cells to normal ones, we examined their ability to divide on soft agar and the ability of cells to "avoid" contact inhibition. According to the results of both tests presented in Fig. 9 and 10, it can be concluded that cells under SELENOT-knockout did not show signs of conversion into normal ones, as their proliferative properties and division rate did not differ from the that of the untreated control cells.

3. Discussion

We studied the effect of suppression (to approximately 10%) of SELENOT mRNA expression (approximately 10 times) and protein content of SELENOT is quite high in them compared in two human cancer cell lines. Moreover, these two cell lines A-172 and Caco-2, were found most suitable for efficient transduction with lentiviral particles. The main objectives of this study were (i) to explore explanation of the role of SELENOT in the regulation of molecular mechanisms of ER-stress caused by selenium containing as well as non-selenium sources of ER-stress, (ii) to study the effect of SELENOT-knockdown on the expression of its previously identified physiological partners, other ER-resident selenoproteins, selenoproteins, enzymes that are key regulators of redox homeostasis, such as glutathione peroxidases and thioredoxin reductases. (iii) In addition, a series of experiments was carried out, the purpose of which was to find out whether suppression of SELENOT mRNA expression affects conversion to normal cells as detected by enhanced features of normal cells in them. The main goal of this study was to explore the role of SOLENOT in the ER with and without ER-stress in the processes of carcinogenesis, using as model cell lines of two widespread types of cancer, glioblastoma and colorectal adenocarcinoma. The choice of these cell lines was made because the mRNA expression and protein content in them is quite high compared to other available cell lines. In addition, these cells of both lines were found to be most suitable for efficient transduction, since the percentage of transduced cells was over 90%.

We selected MSA and SS as selenium-containing ER-stress sources for A-172 and Caco-2 [13,14]. The choice of these ER-stress inducers was not made by chance. We previously carried out studies on cytotoxic effects of MSA and SS in these cell lines and analysed the expression of SELENOT at various concentrations. It was found that when both cell lines were treated with 0.1 μM MSA or SS, a slight increase in SELENOT expression was observed. In addition, when these cell lines were treated with 0.1 μM MSA or SS, a greater percentage of cells were in a state of apoptosis compared to 0.01 μM concentrations of these compounds. In addition, earlier, a similar series of experiments was carried out using the well-known non-selenium ER-stress inducer DTT; it was shown that DTT in Caco-2 cells promoted an increase in the expression of SELENOT.

It is known that the cytotoxic effect of selected ER-stress inducers is mediated by various molecular mechanisms, primarily due to the production of various metabolites. The final metabolite of SS is hydrogen selenide, which, after being converted into selenophosphate, actively participates in the synthesis of selenoproteins through the interaction with selenocysteine tRNA. The active metabolite of

MSA is methylselenol. The manifold antitumor mechanisms of MSA include: glutathione-dependent induction of lipid peroxidation should be distinguished [15], inhibition of the PI3K/AKT/mTOR pathway and activation of FOXO proteins [16], inhibition of the activity of deacetylase and DNA methyltransferase [17], inhibition of angiogenesis by suppressing β 3-integrin and interrupting its clustering [18]. DTT is a strong reducing agent of disulfide bonds in proteins due to two consecutive exchanges of thiol disulfides between cysteine residues in proteins. In addition, it is known that DTT is a pharmaceutical nonspecific inducers of ER-stress, since it blocks the formation of disulfide bonds in all proteins, including those newly synthesized in the cytosol. We selected this agent as a non-selenium source of ER stress, first of all, to establish the presence/ absence of a specific effect of MSA and SS on the expression of the studied genes, especially selenoproteins, under SELENOT-knockdown.

In the course of this work, it was established that SELENOT-knockdown does not affect either the viability and proliferative properties of A-172 and Caco-2 cells, and does not contribute to their acquisition of the properties of normal cells.

SELENOT-knockdown had practically no effect on the mRNA expression of key ER-stress genes; only when the cells were treated with 1 mM DTT, a slight decrease in the expression of the following genes was observed: GADD34, PUMA, BAX, CHOP and BAK. However, these small fluctuations in the mRNA expression of these genes did not have a conspicuous effect on the pathological state of cells under conditions of prolonged ER-stress as compared to cells in which SELENOT-expression was not disturbed.

According to the results of their study, the greatest SELENOT-knockdown effect was found in the mRNA gene expression and protein content of the selenoprotein SELENOM as well as its two physiological partners AMFR and RNF5 under ER-stress conditions.

According to the results of our studies, it became obvious that among all seven selenium-containing proteins localized in the ER, only DIO2 and SELENOM most vividly reacted to a decrease in mRNA expression of SELENOT in the studied cancer cells. Moreover, DIO2 mRNA expression decreased in the absence of ER-stress and did not change when cells were treated with all three sources of ER-stress. Conversely, the expression of SELENOM mRNA decreased only under ER-stress, especially when cells were treated with 1 mM DTT and 5 mM DTT.

It is difficult to interpret the direct dependence of a decrease in the SELENOT mRNA expression on the expression of DIO2 mRNA; these data require confirmation by other independent approaches. As for SELENOM, this relationship is understandable. First, both proteins have a thioredoxin-like folding, which may explain the similarity of their functional role [19]. Previously, we have shown that, when DU 145, HT-1080 and MCF-7 cancer cells were treated with 0.1 μ M MSA, the expression of SELENOT mRNA changed synchronously with SELENOF, which also, along with SELENOT and SELENOM, have a thioredoxin-like folding, and always asynchronously with the expression of SELENOM mRNA [11]. Therefore, in this case, it would be more logical to have an inverse correlation in the expression of mRNA of both selenoproteins. In general, out of all seven proteins, the function of SELENOM in the regulation of ER-stress and its role

for ER in general remains less clear. In many cancer cells, the expression of its mRNA is high. We have repeatedly shown that its expression reacts to a large extent to the effects of selenium-containing sources of ER-stress [12–14, 20-25]. It is interesting that, like SELENOM protein, mRNA expression also decreased in two proteins that are components of the ERAD system: AMFR and RNF5. Earlier, the same tendency of these proteins upon SELENOT-knockout was observed for endocrine cells [9]. AMFR–E3 ubiquitin–protein ligase mediates polyubiquitinylation of lysine and cysteine residues of target proteins for subsequent proteasome degradation. It was initially identified as a tumor autocrine motility factor receptor that promotes the invasion and metastasis of tumors. RNF5, Ring finger protein 5, is an E3 ubiquitin–protein ligase, knockdown of which significantly reduced AMFR–mediated ubiquitinylation of CFTR, the cystic fibrosis transmembrane conductance regulator [26]. AMFR and RNF5 can be linked to each other via members of the Derlins family proteins (Derlin 1, 2, 3) and function as a Derlin-containing complex for the polyubiquitinylation of a number of proteins. It has been shown that SELENOT is involved in the N–glycosylation of endogenous glycoproteins and is a subunit of the A–type oligosaccharyltransferase complex (OST) [11]. The results of this work are consistent with the previously revealed dependence of a decrease in the mRNA expression of AMFR and RNF5 on a decrease in the mRNA expression of SELENOT [11]. Since a similar pattern of changes in mRNA expression in A–172 and Caco–2 cells under ER–stress was also established for SELENOM, it is possible that this selenoprotein is also involved in the processes associated with cellular protein quality control (PQC) system and proteolytic machinery of cells. However, this remains to be elucidated by further research.

It has been shown that SELENOT interacts with KRTCAP2 (KCP2), STT3A, DDOST (OST 48), but not with STT3B [11]. However, according to our results, the mRNA expression of these proteins remained practically unchanged upon SELENOT–knockdown both under ER–stress and in intact cells.

It has been reported that a decrease in the expression of two important enzymes of the ERAD–system upon SELENOT–knockdown leads to the accumulation of proteins with incorrect folding in the ER lumen, which, apparently, only aggravates ER–stress in cancer A–172 and Caco–2 cells, therefore, no significant changes in cells morphology, redox homeostasis were observed in our experiments.

4. Conclusions

In the course of this work, it was found that SELENOT–knockdown with or without ER stress does not significantly affect viability, proliferative properties, redox homeostasis, the acquisition of signs of normal cells in A172 and Caco-2 cells. Furthermore, SELENOT-knockdown did not significantly affect the expression of ER-stress marker genes and ER-resident selenoproteins, except for DIO2 and SELENOM. At the same time, it had a significant effect in reducing the levels of mRNA expression of AMFR and RNF5, important enzymes of the ERAD system, which can be expected to disrupt its proper functioning, leading to the accumulation of proteins with incorrect folding, thereby to some extent aggravating ER stress.

5. Materials And Methods

5.1. Cell Culture and Reagents

Cell lines A-172 (human glioblastoma) and Caco-2 (human colorectal adenocarcinoma) obtained from «ATCC» (USA) were harvested in a DMEM medium supplemented with 10% fetal bovine serum from «Gibco» (France) and 100 U/ml penicillin, 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. MTT Cell Proliferation Assay Kit and antibodies for Western blots including anti-GAPDH (ab181602), anti-DIO2 (ab77779), anti-SELENOT (ab122587), anti-SELENOM (ab133681) and secondary antibody conjugated to horseradish peroxidase (ab99697) were obtained from «Abcam» (USA), anti-AMFR (PA5-102892) from «Thermo Fisher Scientific» (USA). Noble agar, Puromycin dihydrochloride, SS, MSA, DTT, purchased from «Sigma-Aldrich» (USA), were dissolved in distilled sterile water. ExtractRNA reagent, First-strand cDNA kit, SYBR Green I PCR Master Mix and Lentivirus particles were purchased from «Evrogene» (Russia). Real-time PCR was performed on a CFX384 Touch thermocycler, Bio-Rad (USA).

5.2. Cell Proliferation and Viability Assay

Viability assay was carried out on the counter Countess II FL «Life Technologies» (USA). For this, untreated cells and cells with SELENOT-knockdown were seeded on a 96-well plate at 5500 cells/well and were resuspended in a 1x PBS solution, stained with 0.4% trypan blue, and applied to a glass slide of the counter, where the stained cells were counted.

For this, untreated cells and cells with SELENOT-knockdown were seeded on a 96-well plate at 5500 cells/well. Cells were then incubated for 3 hours with MTT Reagent at 37°C. After incubation, cells were treated with MTT Solvent for 15 min at room temperature. Absorbance was measured at OD=590 nm using a microplate reader.

5.3. Total RNA isolation, reverse transcription and real-time quantitative PCR

Total RNA from control cells and SELENOT-knockdown cells was isolated using the ExtractRNA reagent according to the manufacturer's instructions carried out by Evrogene (Russia). The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized from 2 µg of total RNA using oligo dT primers and MMLV reverse transcriptase. Quantitative Real-time PCRs were performed in a 25µL reaction mixture containing SYBR Green I PCR Master Mix and 300 nM of the appropriate primers (Table 1). The PCR procedure consisted of 95°C for 2 min followed by 40 cycles of 95°C for 1 min, 60°C for 15 s and 72°C for 15 s. Electrophoresis was performed with the PCR products to verify primer specificity and product purity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization, and results were expressed as $2^{-\Delta(\Delta Ct)}$.

5.4. Western blot

Cells were homogenized with Cell Lysis Buffer (100 mM Tris–HCl, pH 8.0, 0.15 mM NaCl, 1 mM EDTA, 1 mM PMSF). The lysates were cleared by centrifugation at 14,000 g for 10 min at 4°C. Proteins were separated by SDS–PAGE on 12.5% polyacrylamide gels, and were transferred onto nitrocellulose membrane. To avoid nonspecific binding of antibodies, the membrane was blocked in 5% dry milk, 1 h at room temperature, and then the membrane was incubated with primary antibodies (1:100–1:500) for 2 h at room temperature. Thereafter, blots were incubated for 1 h with the secondary antibody conjugated to horseradish peroxidase (1:5000) 1 h at room temperature. Immunoreactive bands were visualized by detection of peroxidase activity by DAB staining (0.05% DAB in TBS+10µl 30% hydrogen peroxide).

5.5. Knockdown of SELENOT by RNA interference and transduction with lentiviral particles

Gene knockdown 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/sirnawizard>. For SELENOT, 3 shRNA variants and one negative control (scramble shRNA) were purchased from «Evrogen» (Russia). The delivery of shRNA as part of the pGPV vector containing the P1 promoter for shRNA expression was carried out by chemical transfection (lipofection) using the Lipofectamine 2000 reagent «Invitrogen» (USA). Transfection was performed at the stage of logarithmic growth of cells with a confluence of 60–70% in 24–well plates, the number of cells ranged from $1-2 \times 10^5$. The procedure was performed in serum–free DMEM medium supplemented with 0.5 µg of plasmid DNA and 2 µl of Lipofectamine® 2000 reagent per well. The analysis of the efficiency of transfection was carried out after 48 h using fluorescence microscopy, since the pGPV plasmid contained the gene for the fluorescent protein CopGFP. Evaluation of the knockdown efficiency was carried out using real–time PCR and Western blotting. The most efficient shRNA sequence was used in transduction with lentiviral particles.

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 knockdown after transduction with lentiviral particles was by real–time PCR and Western blotting.

5.6. Soft agar assay

A stock solution of agarose (3.2%) was autoclaved and cooled in a water bath to 38.5°C. Then, the base layer of agarose (0.8%) was prepared and was poured into 1 ml per 30 mm dish and cooled for 5–10 min at 4°C. Next, the top layer of agarose (0.48%) was prepared. Cells (2–3000 per dish), previously removed from the plates with trypsin, were resuspended in 5 ml of culture medium, after which they were mixed with 750 µL of 3.2% stock solution of agarose (38.5°C), carefully pipetted and added 1 ml per plate over the base layer, 1 ml of culture medium was added to the dish on top layer. The cells were incubated in a CO₂ incubator for 10–15 days until the formation of colonies. Growing colonies were stained with 0.005% Crystal violet; cells were preliminarily fixed in 4% formaldehyde? stained for 2 h, after which the agarose, containing the cells, was washed from the dye and the colonies were counted using Zeiss Axio Observer Z1 microscope «Zeiss» (Germany).

5.7. Analysis of the ability of cancer cells to "avoid" contact inhibition

Intercellular contacts, formed by populations of normal cells in two-dimensional culture, suppress further cell proliferation, forming a confluent monolayer. Contact inhibition is lacking in many types of cultured cancer cells. To analyze this ability, the structure of the cell monolayer was disrupted with the tip of a pipette. Control of cell migration to the "wound" area using Zeiss Axio Observer Z1 microscope «Zeiss» (Germany) was carried out after 24, 48 and 72 hours.

5.8. Statistical analysis

Microsoft Excel and GraphPadPrism 5 software (GraphPad, San Diego, CA, USA) was used for data analysis, graph creation, and statistic processing. The concentration of protein was calculated by standard curve plotted with 1 mg/mL of BSA solution. Values are given as the mean \pm SD of at least three independent experiments. Statistical analysis was performed using a one or two-way ANOVA. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. In the analysis of multiple comparisons, the Sidak's correction was used. Differences were considered significant when p-value was <0.05 . Protein expression was quantified using ImageJ program.

Abbreviations

AMFR, autocrine motility factor receptor; ATF4, activating transcription factor-4; ATF6, activating transcription factor-6; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-like protein 4; Bim, Bcl-2-like protein 11; Bcl-2, B cell leukemia/lymphoma 2; CHOP, CCAAT/enhancer-binding protein-homologous protein; DDOST, dolichyl-diphosphooligosaccharide-protein glycosyltransferase; DERL1,2,3, degradation in endoplasmic reticulum protein 1,2,3; DTT, dithiothreitol; ER, endoplasmic reticulum; ERAD, endoplasmic-reticulum-associated protein degradation; GADD34, growth arrest and DNA damage gene; GPX,

glutathione peroxidase; IRE1, inositol–requiring enzyme–1; KRTCAP2, keratinocyte associated protein 2; MAPK3K5, mitogen–activated protein kinase kinase kinase 5; MAPK–8, PERK–Protein kinase–like ER kinase; MSA, methylseleninic acid; Puma, p53 up–regulated modulator of apoptosis; RNF5, ring finger protein 5; SEL, selenoprotein; SS, sodium Selenite; STT3A, STT3 oligosaccharyltransferase complex catalytic subunit A; STT3B, STT3 oligosaccharyltransferase complex catalytic subunit B; TXNRD, thioredoxin reductase; UPR, unfolded protein response; XBP1, X–box binding protein–1.

Declarations

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Author Contributions

E.G.V. coordinated the project and wrote the manuscript, M.V.G. performed experiments with fluorescence microscopy and vitality tests, E.V.B performed western blots and PCR analysis. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Figures

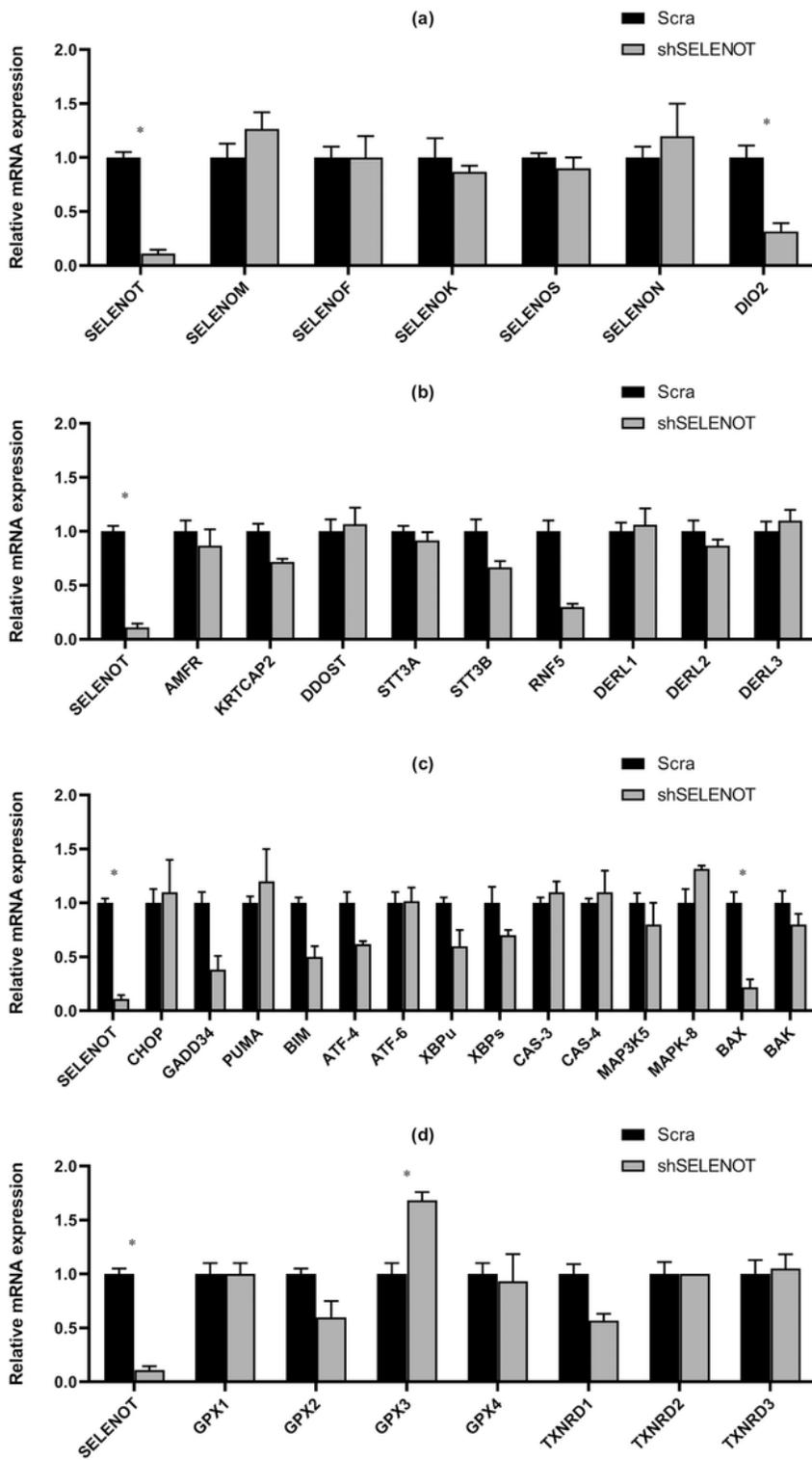


Figure 1

The mRNAs expression of various genes in A-172 cancer cell line after SELENOT-knockdown. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. Comparison scramble shRNA and SELENOT-knockdown,* p-level < 0.05. GAPDH was used as an internal control for normalization. a-the mRNAs expression of ER-resident selenoproteins; b-the mRNAs expression of proteins-partners of SELENOT; c-the mRNAs expression of

genes—markers of ER-stress; d—the mRNAs expression of glutathione peroxidases and thioredoxin reductases.

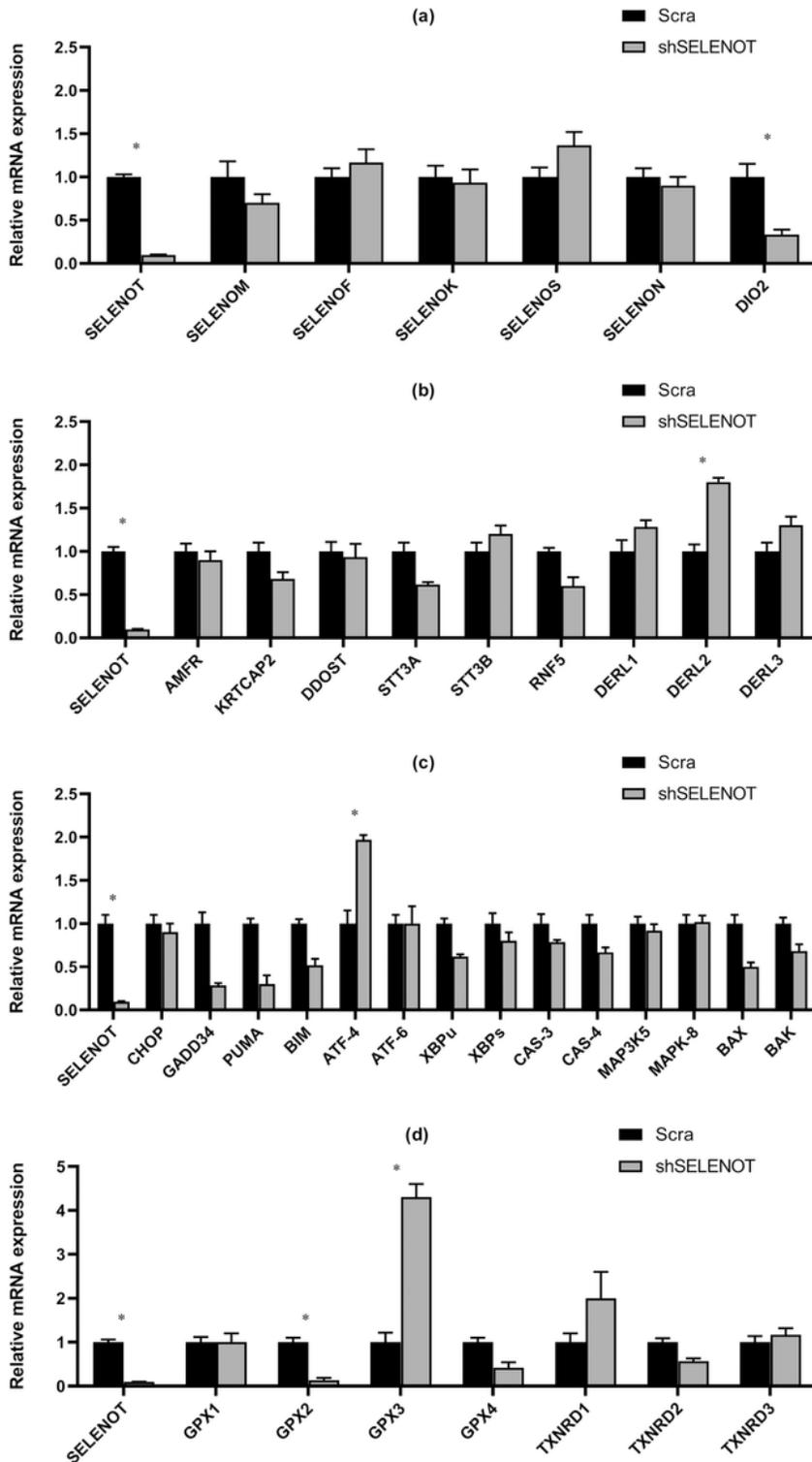


Figure 2

The mRNAs expression of various genes in Caco-2 cancer cell line after SELENOT-knockdown. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. Comparison scramble shRNA and SELENOT-knockdown,* p-level < 0.05.

GAPDH was used as an internal control for normalization. a–the mRNAs expression of ER–resident selenoproteins; b–the mRNAs expression of proteins–partners of SELENOT; c–the mRNAs expression of genes–markers of ER–stress; d–the mRNAs expression of glutathione peroxidases and thioredoxin reductases.

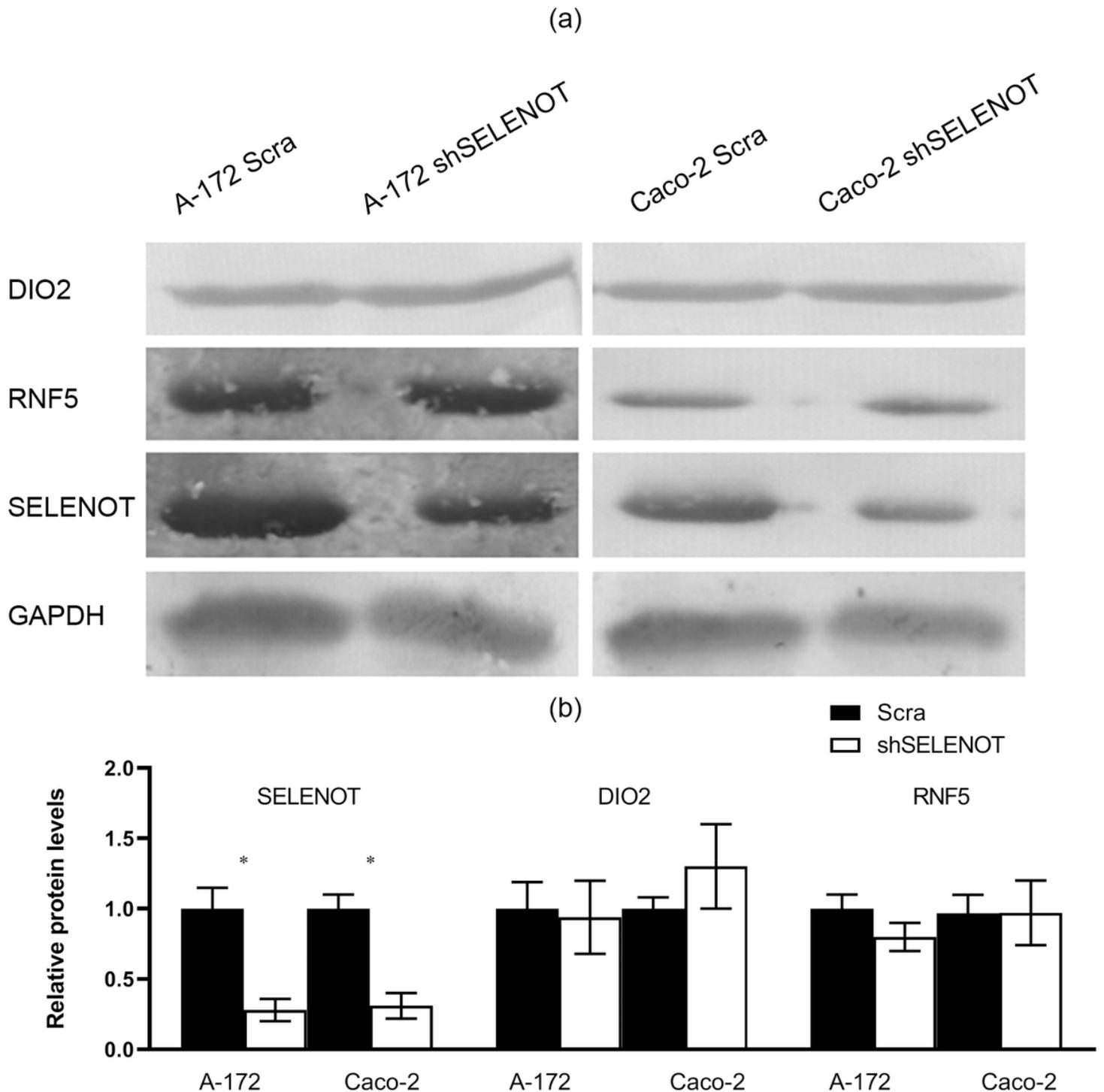


Figure 3

The protein abundance of ER–resident selenoproteins SELENOT, DIO2 and RNF5 in A–172 and Caco–2 cancer cells before and after SELENOT–knockdown. Each value is the mean \pm SD of at least five

independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. Comparison scramble shRNA and SELENOT-knockdown,* p -level < 0.05 . GAPDH was used as an internal control for normalization. Statistical analyses were performed by paired t-test.

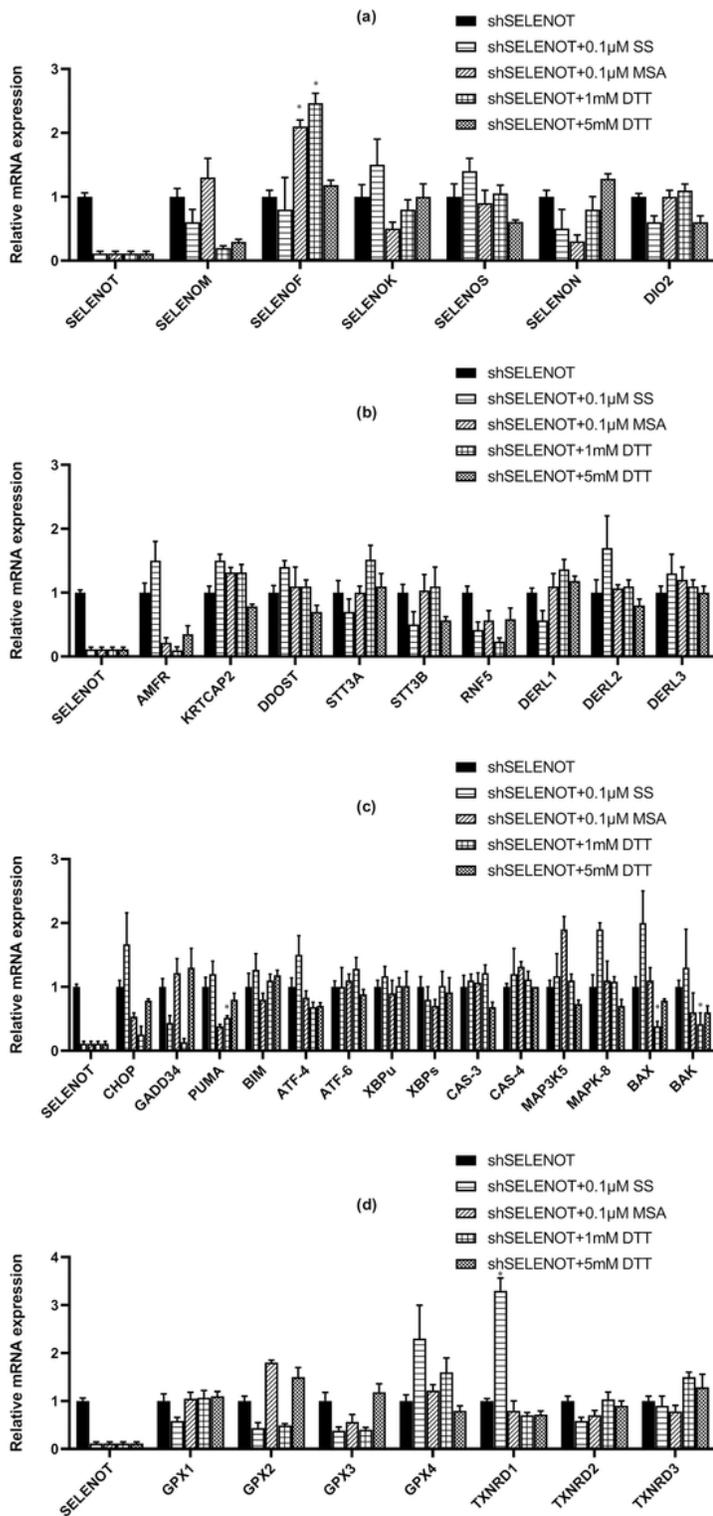


Figure 4

The mRNAs expression of various genes in SELENOT-knockdown A-172 cancer cell line and without SELENOT-knockdown after treatment with 0.1 µM SS, 0.1 µM MSA, 1 mM DTT and 5 mM DTT for 24 h.

Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. GAPDH was used as an internal control for normalization. a–the mRNAs expression of ER–resident selenoproteins; b–the mRNAs expression of proteins–partners of SELENOT; c–the mRNAs expression of genes–markers of ER–stress; d–the mRNAs expression of glutathione peroxidases and thioredoxin reductases.

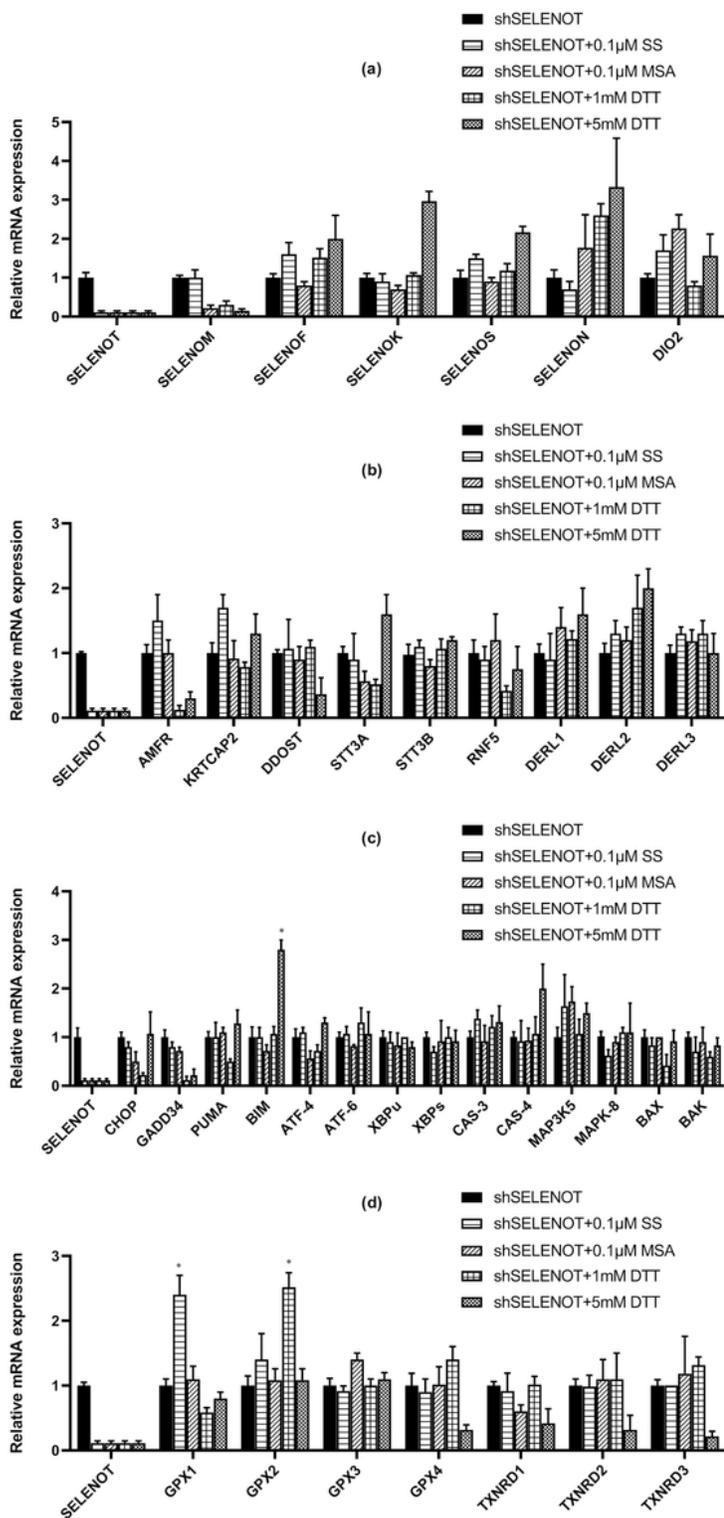
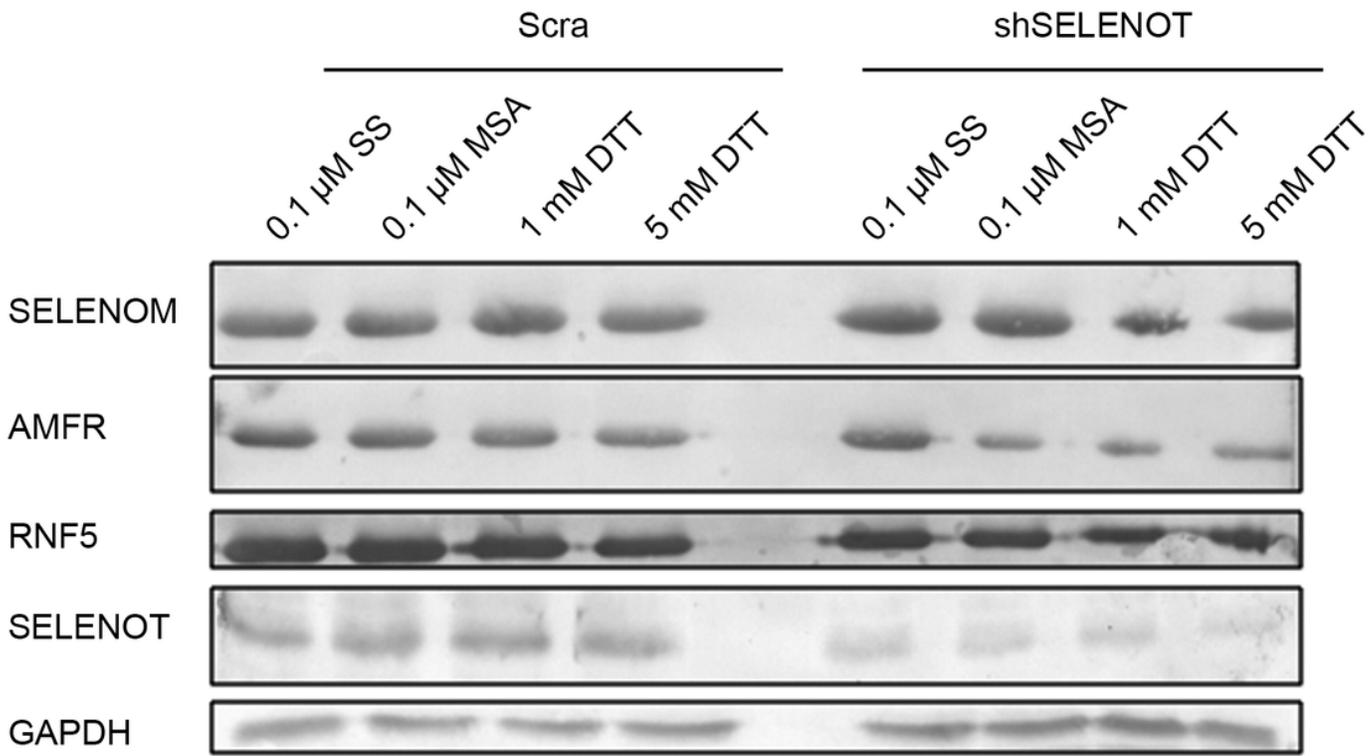


Figure 5

The mRNAs expression of various genes in SELENOT–knockdown Caco–2 cancer cell line and without SELENOT–knockdown after treatment with 0.1 μ M SS, 0.1 μ M MSA, 1 mM DTT and 5 mM DTT for 24 h. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. GAPDH was used as an internal control for normalization. a–the mRNAs expression of ER–resident selenoproteins; b–the mRNAs expression of proteins–partners of SELENOT; c–the mRNAs expression of genes–markers of ER–stress; d–the mRNAs expression of glutathione peroxidases and thioredoxin reductases.

(a)



(b)

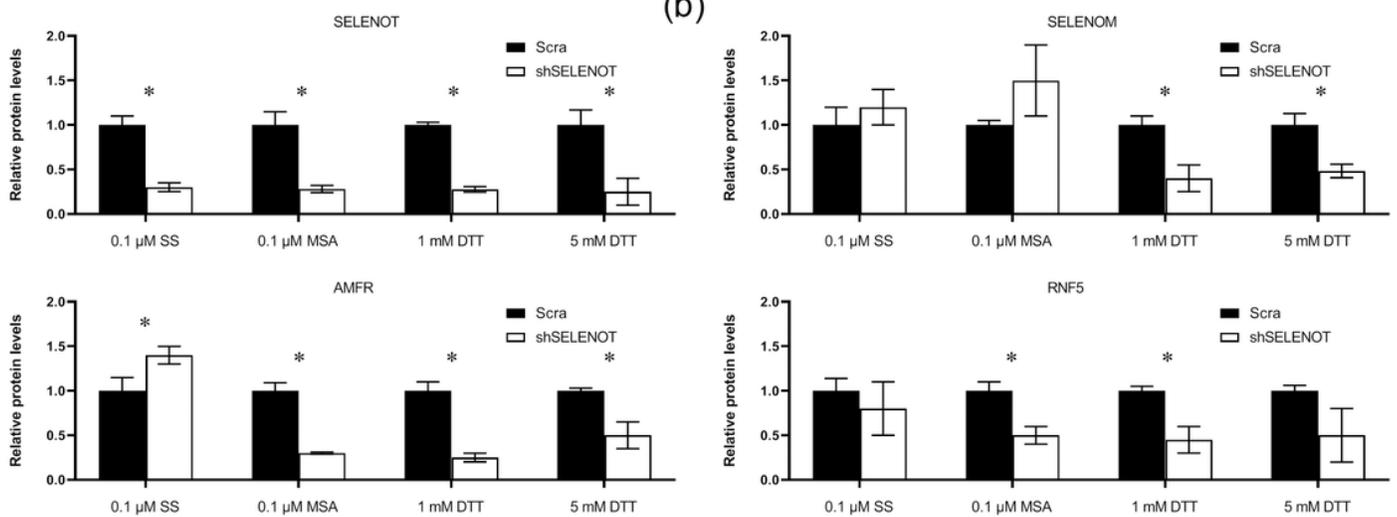


Figure 6

The protein abundance of various genes in SELENOT–knockdown A–172 cancer cell line and without SELENOT–knockdown after treatment with 0.1 μ M SS, 0.1 μ M MSA, 1 mM DTT and 5 mM DTT for 24 h. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired nonparametric Mann-Whitney u-test was used. GAPDH was used as an internal control for normalization. Statistical analyses were performed by paired t–test.

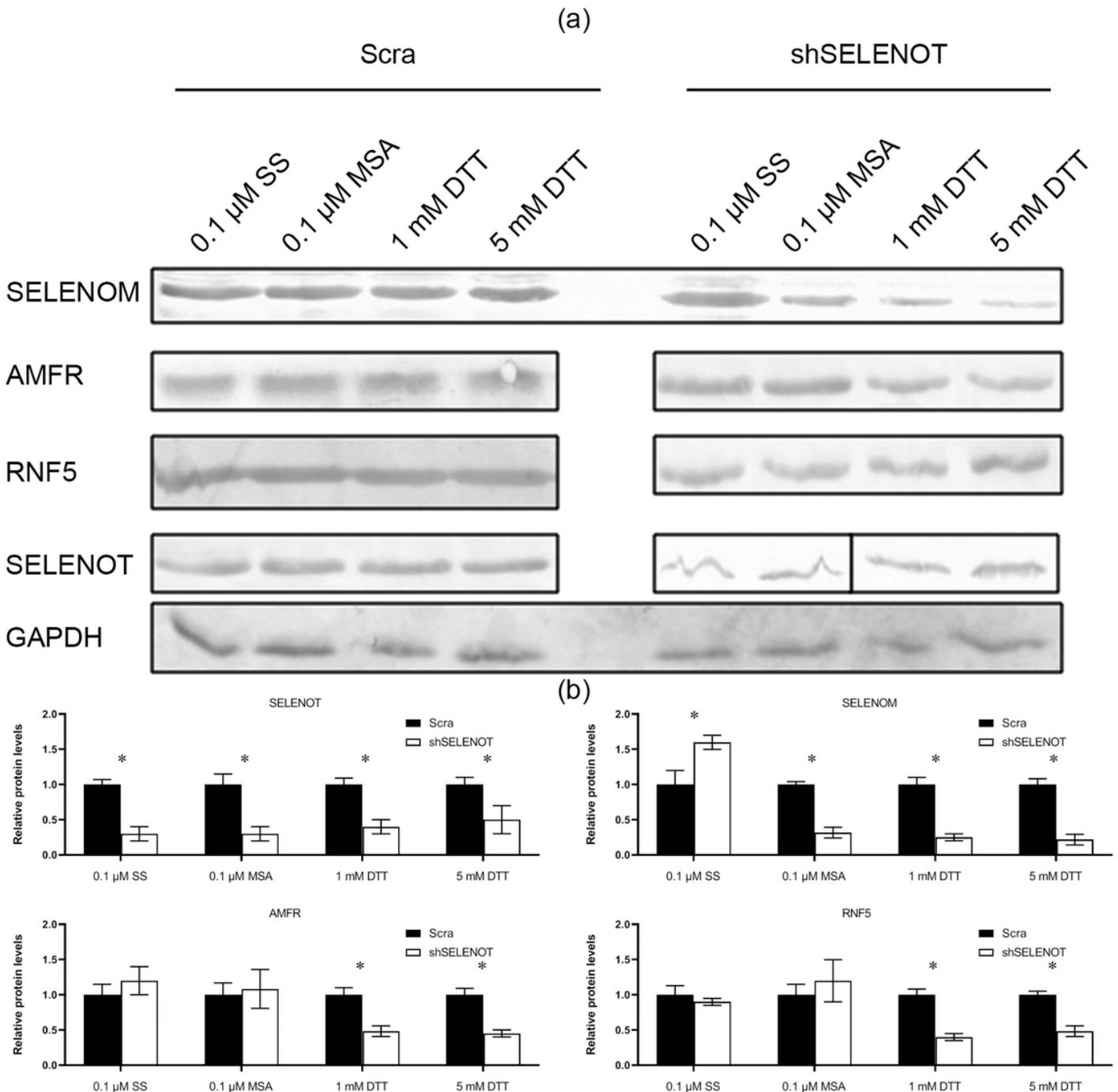


Figure 7

The protein abundance of various genes in SELENOT–knockdown Caco–2 cancer cell line and without SELENOT–knockdown after treatment with 0.1 μ M SS, 0.1 μ M MSA, 1 mM DTT and 5 mM DTT for 24 h. Each value is the mean \pm SD of at least five independent experiments ($n \geq 5$, $p < 0.05$). Unpaired

nonparametric Mann-Whitney u-test was used. GAPDH was used as an internal control for normalization. Statistical analyses were performed by paired t-test.

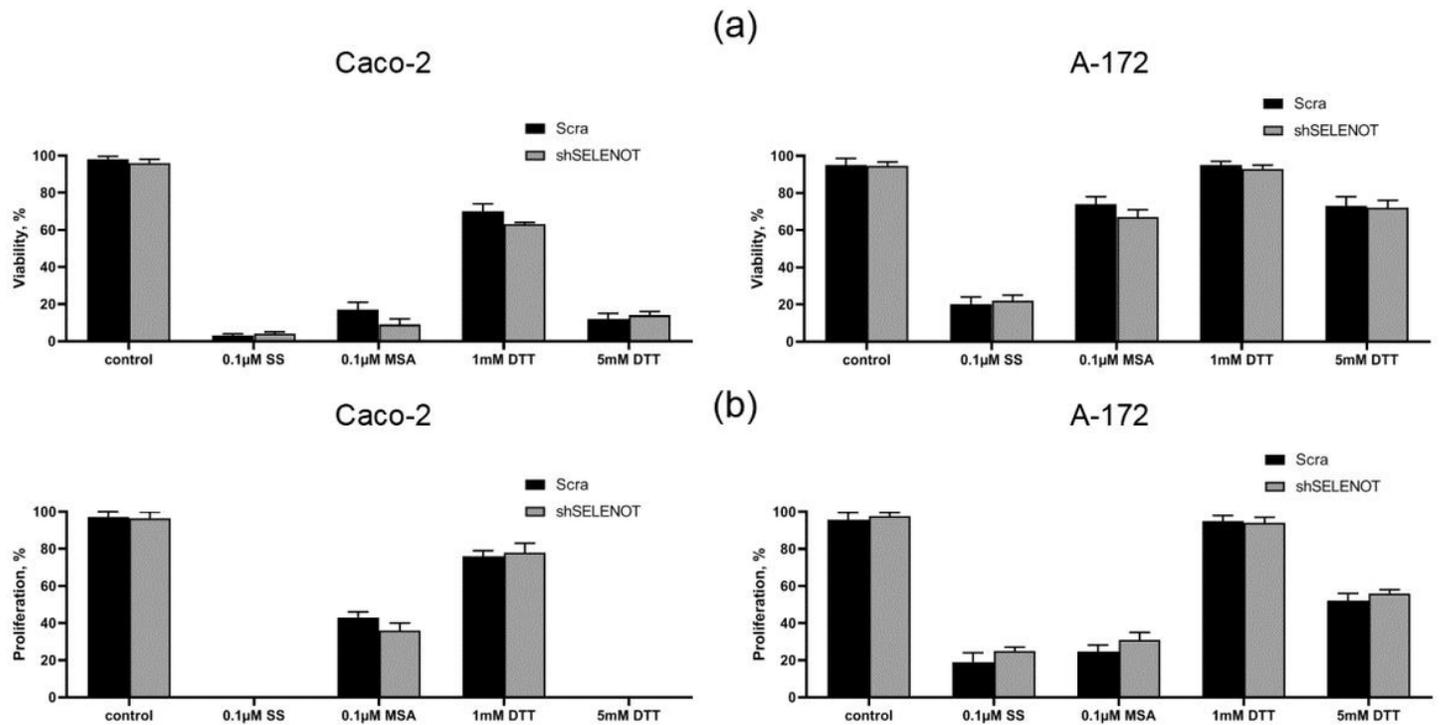


Figure 8

Comparison of the viability of A-172 and Caco-2 cells before and after SELENOT-knockdown after treatment with 0.1 μM SS, 0.1 μM MSA, 1 mM DTT and 5 mM DTT for 24 h. a-viability assay performed with the counter Countess II FL (Life Technologies, USA). Standard deviations were determined by analysis of data from at least three independent experiments, and are indicated by error bars. b-proliferation assay performed by MTT-analysis. The optical density at 590 nm was measured, and the values of the respective untreated cells were defined as 100%. Standard deviations were determined by analysis of data from at least three independent experiments, and are indicated by error bars.

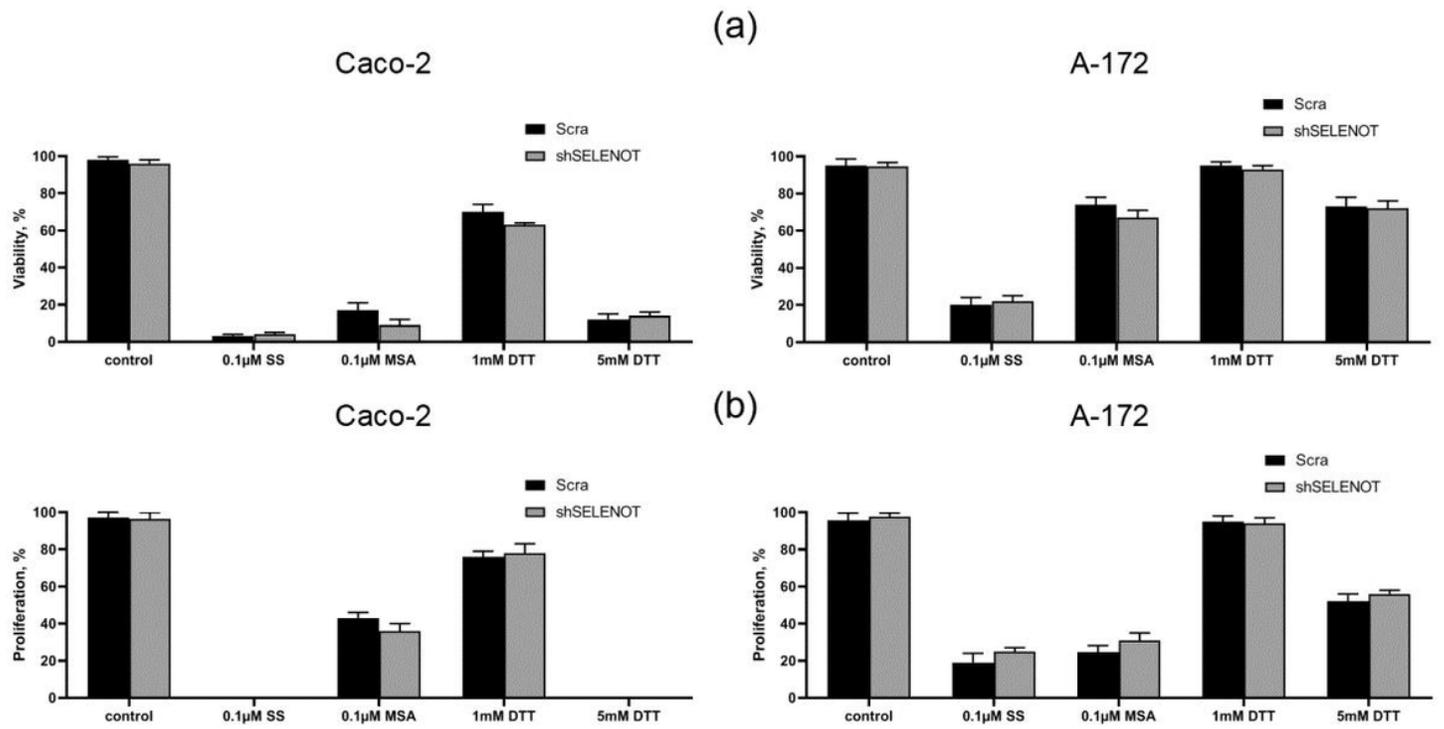


Figure 9

Effects of SELENOT-knockdown on anchorage-independent colony formation in soft agar. a-representative soft agar dishes with stained colonies of A-172 cells; b-representative soft agar dishes with stained colonies of Caco-2 cells

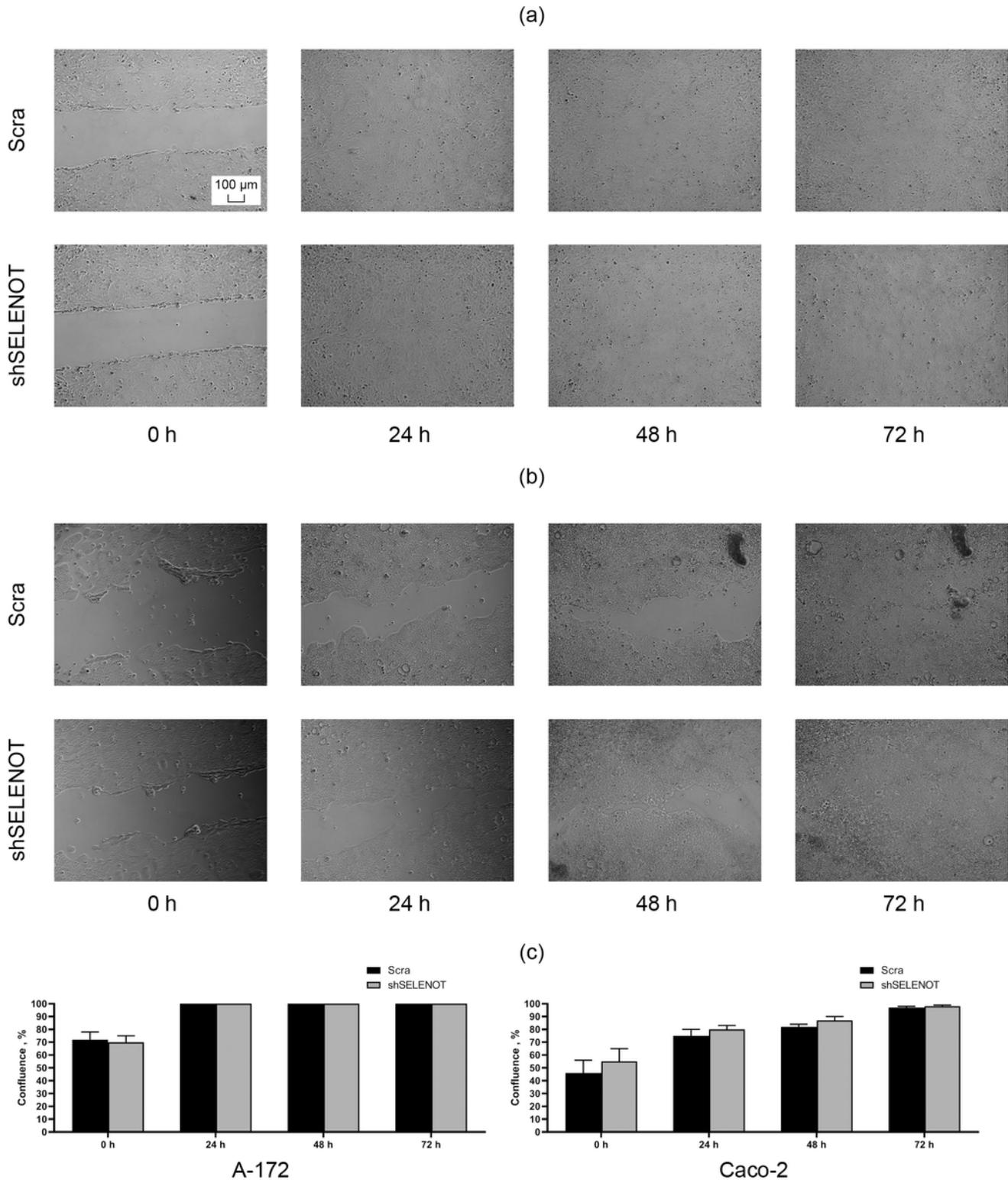


Figure 10

Effects of SELENOT-knockdown on cell migration by the wound healing assay. a–representative cell migration assay for A-172 cells after ; b–representative cell migration assay for Caco-2 cells; representative soft agar dishes with stained colonies of Caco-2 cells. Cell migration assays were performed 24, 48 and 72 hours after monolayer disruption.

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