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HERPUD1, a member of the Endoplasmic Reticulum Protein Quality Control Mechanism, may be a Good Target for Suppressing Tumorigenesis in Breast Cancer Cells

Short Title: HERPUD1 and Breast Cancer

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ABSTRACT
Objectives: Breast cancer is the most frequently diagnosed cancer type in women, and it is the second leading cause of cancer-related death in women. Recent studies highlight the importance of the Endoplasmic reticulum (ER) protein quality control (ERQC) mechanism for the survival of many cancers and it also has been recommended as a good target for the treatment of many cancer types. Homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) functions as one of the main components of ER-associated degradation (ERAD), which is an ER-resident protein quality mechanism. Today, the association of HERPUD1 with breast carcinogenesis is still not fully understood. Herein, we evaluate the possibility of HERPUD1 as a potential therapeutic target for breast cancer.
Materials and Methods: The effect of HERPUD1 silencing on epithelial-mesenchymal transition, angiogenesis and cell cycle proteins was analyzed by immunoblotting studies. To test the role of HERPUD1 on tumorigenic features, WST-1 based cell proliferation assay, wound-healing assay, 2D colony formation assay and Boyden-Chamber invasion assay were performed in MCF-7 cells. The statistical significance of the differences between the groups was determined by Student t-test.
Results: Our results showed that suppression of HERPUD1 expression led to decreased cell cycle-related protein levels including Cyclin A2, Cyclin B1 and Cyclin E1 in MCF-7 cells. Also, silencing of HERPUD1 was remarkably decreased expression levels of epithelial-mesenchymal transition-related N-cadherin and angiogenesis marker, VEGF-A. Moreover, we determined that significantly decreased the cell proliferation, migration, invasion and colony formation in HERPUD1 silenced MCF-7 cells.
Discussion and Conclusion: HERPUD1 may have the potential to be an effective target for biotechnological and pharmacological targeting strategies to be developed for the treatment of breast cancer.
Keywords: Breast Cancer, ERAD, HERPUD1.

Conflict of Interest
The authors declare no conflict of interest
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INTRODUCTION
According to statistical studies conducted in the United States, breast cancer is the most frequently diagnosed cancer type in women, and it is the second leading cause of cancer-related death in women.[1] It is known that it is

a hormonal regulation-dependent cancer type because 70% of breast cancer cells contain estrogen receptors (ER) and/or progesterone receptors (PR). The most commonly used method in treating breast cancer is conservative surgery. Moreover, depending on the receptor expression profiles, alternative approaches such as tamoxifen, an estrogen receptor modulator can be used as a treatment option. Today, the hormonal treatment approach can be chosen based on the receptor status of the primary tumor in patients.[2,3] Although alternative treatment approaches have been introduced through developing technology and new drug candidate molecules, there is an ongoing need to understand the molecular pathogenesis of breast cancer better and to identify the molecular focuses that support the carcinogenesis process.

Numerous studies have reported that ER stress is activated at high levels in hematopoietic (leukemia, lymphoma, and myeloma) and solid tumors including stomach, colon, esophagus, lung, prostate, pancreas, liver glioblastoma, and breast cancer as well.[4] It has been determined that protein levels associated with molecular chaperones which are involved in the protein quality control in the ER and molecular mechanisms that mediate the removal of misfolded proteins to prevent proteotoxicity are expressed at high levels in many cancer types.[5] This process provides the ability to adapt to rapidly proliferating cancer cells against increased proteome activity and is necessary for providing cellular basal needs and preventing apoptotic cell death signals.[4]

Endoplasmic reticulum (ER) is the largest organelle of the cell and functions as a center for crucial biological functions such as protein synthesis, transport, protein folding, lipid and steroid synthesis, carbohydrate metabolism and Ca^{+2} storage.[6] Protein folding is not a faultless process, it is known that one-third of newly synthesized proteins in cells are misfolded.[7] Although proteins are folded properly, they frequently undergo conformational deterioration inside the cell as they are constantly exposed to cellular stresses such as heat and oxidative stress. This situation leads to the re-release of hydrophobic residues in proteins that result in the formation of protein aggregates and thus cellular proteotoxicity.[7] Accumulation of misfolded proteins in the ER lumen triggers a process called “ER stress” by lowering free chaperone levels. The only possible way for cells to overcome this stress is by increasing the protein folding capacity of the ER and eliminating the misfolded proteins by directing them to the protein degradation process.[8]

Numerous molecular mechanisms are triggered to overcome ER stress in cells, and one of the ways to compete for potentially proteotoxic proteins with folding defects is the mechanism known as Endoplasmic Reticulum-associated degradation (ERAD).[9] ERAD has multiple steps, including substrate recognition, initiation of retrotranslocation (translocation in the lipid bilayer), ubiquitination, retrotranslocation, targeting 26S proteasome, and proteasomal degradation. Targeting for proteasomal degradation is a highly controlled sophisticated cellular system that diverse protein units take part in this process according to the type of targeted protein.[10]

Homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) is a 54 kDa protein located in the ER membrane, and it has been determined to play a role in the retrotranslocation step, which is expressed as translocation of misfolded proteins from the ER to the cytosol, to direct 26S proteasome-mediated degradation for the elimination of misfolded proteins.[11–13] To date, there is no identified enzymatic activity of HERPUD1 and it has been shown that it regulates ERAD by interacting with proteins involved in the retrotranslocation, ubiquitination, and degradation of misfolded proteins.[14–16] HERPUD1; It is one of the key components involved in stabilizing the ERAD multiprotein complex and effectively directing misfolded proteins to degradation, and is thought to be one of the major components of ERAD.[14,17] In the interaction analyses, it was determined that HEDPUD1 interacted in the ER membrane with Hrd1/SYVN1, an E3 ligase enzyme responsible for protein ubiquitination required to target misfolded proteins to proteasomal degradation, ubiquitin molecule, SEL1L, OS-9 acting as a lectin, and Derlin1, which is proposed as a component of the channel complex.[6,18] HERPUD1 expression is widely distributed in all tissues. Especially, high levels of its expression have been reported in tissues with advanced secretory ability, such as the pancreas.[14] This feature of HERPUD1 suggests that it may have important roles for tissues with high secretory properties such as the breast and prostate.

There is very limited data in the literature associating HERPUD1 with the carcinogenesis process. A study published in 2002 showed that HERPUD1 gene expression in prostate cancer cells is induced by androgens. Studies conducted with patient samples determined that HERPUD1 levels were significantly suppressed in cancer group samples compared to healthy tissues. In these studies, it has been suggested that HERPUD1 may have a function of suppressing tumorigenesis.[19] Also, it was determined that apoptotic cell death was induced in prostate cells overexpressing HERPUD1.[20] In another study, in doxorubicin-resistant breast cancer cell lines it was shown that oxidative stress formation and cell adaptation genes were modulated by metformin administration and HERPUD1 was up-regulated among these genes.[21]

In the present study, the effect of suppression of HERPUD1 expression on tumorigenic properties was evaluated in MCF-7 human metastatic breast adenocarcinoma cell line. In this context, HERPUD1 expression was silenced by DsiRNA in MCF-7 cells, and its effects on cell proliferation, migration, invasion and colony formation abilities, as well as its relations with cell cycle and epithelial-mesenchymal transition (EMT), were evaluated. Our results showed that suppression of HERPUD1 expression led to cell cycle arrest in MCF-7 cells and

suppressed EMT. In addition, functional test data indicated that silencing of HERPUD1 in MCF-7 cells strongly limited the cell proliferation, migration-invasion and colony formation abilities.

MATERIALS AND METHODS

Materials

All cell culture grade reagents including growth media, fetal bovine serum (FBS), L-Glutamine and additional growth requirements were obtained from Biological Industries. Rabbit polyclonal HERP1 (10813-1-AP), N-cadherin (22018-1-AP), and E-cadherin (20874-1-AP) antibodies were obtained from Proteintech. Rabbit polyclonal cyclin E1 (#20808), cyclin A1 (#91500), and cyclin B1 (CST #12231) antibodies were obtained from Cell Signaling Technology. Rabbit polyclonal VEGFA (#E-AB-53277) antibody was obtained from ElabScience. Mouse monoclonal beta-actin antibody (#A5316) was purchased from Sigma Aldrich. HRP-conjugated goat anti-mouse (#31430) or goat anti-rabbit (#31460) IgG (H+L) was purchased from Pierce.

Cell culture

Human metastatic breast adenocarcinoma cell line, MCF-7 was purchased from American Type Tissue Culture (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biological Industries) medium with 10% FBS and 2 mM L-glutamine (Gibco™) under a humidified atmosphere of 5% CO2 and 95% air at a constant temperature of 37 °C, which is conventional cell culture conditions. No antibiotics were used during the propagation of cells. The absence of mycoplasma contamination was routinely confirmed by using MycoAlert™ Mycoplasma Detection Kit (Lonza).

siRNA Transfection

Negative Control DsiRNA and HERPUD1 DsiRNA were ordered from Integrated DNA Technologies (IDT). siRNA transfection was performed by Xfect RNA Transfection agent (Takara) according to the manufacturer's instructions. MCF-7 cells were seeded in 6-well cell culture dishes ($1.5x10^6$ cells/well) and after 24 hours the transfection process was started. Cells were transfected with HERPUD1 DsiRNA or control DsiRNA at indicated doses.

RNA isolation, cDNA synthesis and quantitative real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated by NEB Monoarch™ miniprep total RNA isolation kit according to the manufacturer's instructions (New England Biolabs). Quantification of the isolated RNAs was determined by using micro-volume spectrophotometer (MySPEC, VWR). 1 µg RNA was reverse transcribed by iScript™ cDNA synthesis kit following to the manufacturer's instructions (25 °C for 5 min, 46 °C, 20 min and 95 °C for 5 min) (Bio-Rad). Gene expression of HERPUD1 was analyzed by using iTaq Universal SYBR Green reaction mix in CFX96 Real-time PCR instrument (Bio-Rad). The protocol steps were followed according to the manufacturer's instructions. PCR primer sequences are available upon request. The relative gene expression was normalized to GAPDH. Relative quantification was analyzed by $2^{-\Delta\Delta Ct}$ method. In addition, melting-curve analyzes were performed in the last step of the qRT-PCR process to test the specificity of the PCR reactions.

Protein isolation and Western blotting

Protein isolation and immunoblotting studies were carried out as previously reported.[22] MCF-7 cells were lysed with ice-cold RIPA buffer, samples were centrifuged at 14,000 r.p.m for 20 min at 4°C and the supernatant was stored. The total protein concentration was measured by bicinchoninic acid (BCA) method (Takara). Approximately 30 µg protein samples were loaded on hand-cast SDS-PAGE gels and electrophoretically separated and then proteins were transferred to the PVDF membrane. Following the transfer process, blocking, primary antibody application, washing, HRP-conjugated secondary antibody application, washing and monitoring of the proteins steps were followed respectively. Protein bands were monitored by using Clarity Western ECL substrate solution in ChemiDoc XRS+ (Bio-Rad) system.

Measurement of cell proliferation

The cell proliferation rates were performed by WST-1-based cell proliferation assay according to the manufacturer's instructions (Takara). The cells were seeded in 96-well plate (5000 cells/wells). To measure the cell proliferation, 10 µl/well premix WST-1 reactive was applied and cells were incubated for 2 hours under the conventional cell culture conditions. An absorbance reading at a wavelength of 450 nm was performed on a microplate reader (BioTek, Epoch 2). 24- and 48-hour measurements were taken. The results were graphed to form proliferation curves.

Colony formation assay

Plate colony formation assay was performed as described before.[22] MCF-7 cells were seeded in 6-well cell culture dishes with 1000 cells/ml and grown under conventional culture conditions for 72 hours. At the end of the experiment, the cells were washed twice with PBS, and the colonies were fixed with alcohol and staining with crystal violet solution (Sigma Aldrich).

Wound-healing assay

Wound-healing assay was achieved as mentioned before.[22] MCF-7 cells were seeded in 12-well cell culture dishes with $3.5x10^5$ cells/well and grown under conventional cell culture conditions. After 24 hours, the wounds were formed and washed twice with PBS. Following the cells were grown in a culture medium for 72 hours. % of wound closure rates were analyzed with ImageJ software (http://imagej.nih.gov/ij/).

Invasion assay

Boyden-chamber invasion assay was performed as described before.[22] Transwells (Sarstedt) with a size of 8 µm pores were coated with matrigel. In the upper part of the transwell, 10,000 cells were seeded in the serum-free DMEM. In the lower section, a standard DMEM medium with FBS was added. The cells were incubated under conventional culture conditions for 72 hours. The cells invaded from permeable Transwell's pores were fixed and stained with a crystal violet solution (Sigma Aldrich). Counting was performed by photographing the invaded cells using Sunny SopTop microscope and camera system.

Statistical Analysis

The results were presented as mean ± standard deviation (SD). The statistical significance of the differences between the groups was determined by the Student t-test with a minimum confidence interval of 95% using GraphPad Prism 7 software. The value of $p < 0.05$ was considered significant.

RESULTS

Determination of optimum HERPUD1 DsiRNA concentration in MCF-7 cells.

To examine the role of HERPUD1 protein on the carcinogenesis process in human breast adenocarcinoma cell line MCF-7 cells, we aimed to suppress the HERPUD1 expression by using DsiRNA. To determine the optimum HERPUD1 DsiRNA concentration, we tested the various doses of HERPUD1 DsiRNA at 1, 2 and 5 nM for 72 hours. As a control group, cells were transfected with a control DsiRNA at 5 nM concentration, which is known not to target any mRNA. The efficiency of silencing of HERPUD1 expression was tested at the mRNA and protein level by qRT-PCR and immunoblotting assay respectively **(Figure 1a, b).** Our qRT-PCR results showed that the optimum HERPUD1 DsiRNA concentration was 2 nM, which reduced HERPUD1 mRNA expression by 50% compared to the control DsiRNA administered group **(Figure 1a).** Consistent with the qRT-PCR results, 2 nM HERPUD1 DsiRNA concentration efficiently suppressed the HERPUD1 protein expression by 50% compared to the control DsiRNA transfected group. Our findings indicated that the optimum DsiRNA concentration for silencing of HERPUD1 expression in MCF-7 cells was 2 nM **(Figure 1b).**

Investigation of the effects of silencing of HERPUD1 on epithelial-mesenchymal transformation in MCF-7 cells.

We analyzed the protein levels of N-cadherin and E-cadherin in HERPUD1 silenced MCF-7 cells, which are considered markers associated with epithelial-mesenchymal transformation by immunoblotting. In addition, we tested the expression level of VEGF-A protein levels, which is associated with the angiogenesis process in cancer cells. Our results showed that silencing of HERPUD1 remarkably decreased N-cadherin protein levels compared to the control group. However, there was no significant change in E-cadherin levels compared to the control group **(Figure 2).** Silencing of HERPUD1 strongly decreased the VEGF-A protein expression levels compared to the control group **(Figure 2)**.

Evaluation of the effects of silencing of HERPUD1 on some cell cycle control proteins in MCF-7 cells.

Cyclin proteins are critical regulators for the uninterrupted progression of proteomic and genomic rearrangements throughout the cell cycle and are a family of proteins that activate specific cyclin-dependent kinases during phase transitions.[23,24] To understand the possible role of HERPUD1 protein on the cell cycle in MCF-7 cells, we examined the changes in Cyclin A2, Cyclin B1 and Cyclin E1 protein levels in cells with suppressed DsiRNA-mediated HERPUD1 expression by immunoblotting. Our results showed that silencing of HERPUD1 expression remarkably decreased the protein level of all tested cyclin proteins compared to the control group **(Figure 3)**.

Determination of the effects of the suppression of HERPUD1 expression on MCF-7 cell proliferation.

WST-1-based cell proliferation analysis was performed to evaluate the effects of silencing of HERPUD1 in MCF-7 cells on the proliferation of breast cancer cells. Our results showed that the proliferation of HERPUD1 DsiRNA transfected MCF-7 cells was significantly suppressed compared to the control group **(Figure 4)**.

Investigation of the effects of the silencing of HERPUD1 on the wound-healing ability of MCF-7 cells.

The wound healing assay model is a relatively easy and cost-effective tool that is often used to evaluate the migration ability of cancer cells *in vitro*. Therefore, we used this assay system to test the association of HERPUD1 with the migration ability of MCF-7 cells. Our results showed that the migration of MCF-7 cells was significantly decreased by silencing HERPUD1 compared to the control DsiRNA group **(Fig. 5a, b)**.

Testing the effects of silencing of HERPUD1 on the colonial growth ability of MCF-7 cells.

Acquiring the colonial growth ability of cancer cells is accepted as an important sign of the carcinogenesis process at the cellular level.[25] Therefore, to evaluate the relationship of HERPUD1 with tumorigenesis in breast cancer cells, we examined the effects of silencing of HERPUD1 expression on colony formation ability in MCF-7 cells. Our results indicated that DsiRNA-mediated silencing of HERPUD1 expression significantly decreased the colony growth ability of MCF-7 cells compared to the control DsiRNA transfected group **(Figure 6a, b)**.

Evaluation of the effects of HERPUD1 silencing on the invasion features of MCF-7 cells.

Invasion is considered one of the distinguishing features of cancer cells and is expressed as the spread of cancerous cells to neighboring tissues or distant tissues and organs. Invasion is an important factor leading to the spread of cancer cells and limiting their treatment efficacy.[26] The effects of silencing of HERPUD1 expression in MCF-7 cells on invasiveness were evaluated by a matrigel-coated Boyden-chamber transwell assay. Our results indicated that DsiRNA-mediated suppression of HERPUD1 expression significantly reduced the invasion capability of MCF-7 cells compared to the control group **(Figure 7a, b)**.

DISCUSSION

HERPUD1 has a cytoplasm-facing N- and C-terminal regions and is located in the ER. Also, it can be induced by ER stress and homocysteine.[14] HERPUD1 has been proposed as one of the major components of ERAD, which acts as a structure-scaffolding protein in the ER membrane. HERPUD1 acts as a shuttle protein in the retrotranslocation step of ERAD and has no known enzyme activity. It is involved in the regulation of ERAD by interacting with proteins involved in ubiquitination and degradation processes in the retrotranslocation step of ERAD.[16] Also, HERPUD1 controls the proteasomal degradation of the inositol 1,4,5-triphosphate receptor (ITPR) and ryanodine receptor (RYR) proteins which is a Ca^{+2} channel, and has a critical role in Ca^{+2} homeostasis in this way.[27] HERPUD1 exhibits a wide expression profile in all tissues and it has been reported to be expressed at high levels in tissues with high secretory ability, such as the pancreas.[14] This finding suggests that members of the ER protein quality control mechanism, an important mechanism for transition to the secretory pathway of which HERPUD1 is a member, may have important roles in other healthy and cancerous tissues with high secretion ability, such as the breast and prostate.

Herein, we examined the effects of the silencing of HERPUD1 on the tumorigenic properties of MCF-7 breast cancer cells. There is very limited data in the literature linking HERPUD1 to the carcinogenesis process. In studies conducted with samples of the prostate cancer patient, it was reported that HERPUD1 expression was suppressed to a great extent in cancer group samples compared to healthy group samples.[19] In another study, it was shown that the administration of metformin in doxorubicin-resistant breast cancer cell lines modulates oxidative stress generation and cell adaptation genes. Among these identified genes, HERPUD1 has been reported to be up-regulated. [21] Today, the functional effects of suppressing HERPUD1 expression in breast cancer cells are still unknown. Our data show that DsiRNA-mediated silencing of HERPUD1 significantly limits the proliferative ability of MCF-7 cells compared to the control DsiRNA-treated group **(Figure 4)**. In our microscopic examinations during this process, we did not find any sign of cell death in HERPUD1 silenced MCF-7 cells. Since EMT parameters are important in the evaluation of the tumorigenic capacity of cancer cells, we examined the E-cadherin and N-cadherin conversion and also changes in VEGF-A, which is associated with angiogenesis.[28,29] Our results showed that N-cadherin levels were markedly reduced by the silencing of HERPUD1. Also, there was no significant change in E-cadherin levels. Similar to N-cadherin results, VEGF-A levels were also decreased in the HERPUD1 silenced group **(Figure 2a)**. The ER protein quality control mechanism is known to control levels of EMT proteins, tumor suppressors, and oncogenic proteins.[30] These results suggest that ERAD is directly or indirectly involved in the regulation of N-cadherin and VEGF-A levels in breast cancer cells.

In our trials where we tested the effect of HERPUD1 on the cell cycle, we found that suppression of HERPUD1 causes a decrease in cyclin A2, B1 and E2 levels **(Figure 2b)**. Cyclin E1 controls the progression of the G1 phase to the S phase.[31] Some retrospective studies have shown an association between high cyclin E levels and an increased risk of death from breast cancer.[32] Cyclin A2 has been proposed as the bridge between cell cycle and invasion in cancer cells and it has been reported to be expressed at high levels in various human tumors.[33,34] However, it does not always correlate directly with the degree of tumor aggressiveness.[35] Cyclin B1 plays a regulatory role in the transition from the G2 phase to mitosis.[36] It has been reported that cyclin B1 is overexpressed in primary breast, gastric and colorectal cancer cells. [37–39] Our results showed that silencing of HERPUD1 remarkably decreased the levels of all tested cyclins compared to the control group **(Figure 2b)**. This result suggests that HERPUD1 may be directly related to the regulation of the steady-state level of cell cycle phase transition proteins.

Advanced colony formation and increased migration-invasion ability are considered to be hallmarks of the process of tumorigenesis.[25] To test the role of HERPUD1 with tumorigenic properties in breast cancer cells, wound healing, 2D colony formation, and Boyden-chamber invasion assay were performed. Our data indicated that the proliferation, colonial growth and migration-invasion ability of MCF-7 cells was significantly reduced by silencing of HERPUD1 **(Figure 4, 5, 6, 7)**.

Considering that the ERAD mechanism, which functions through large protein complexes, varies according to the characteristics of the substrate molecule to be targeted to the proteasome, it is extremely valuable to investigate the importance of HERPUD1 in breast cancer cells. Collectively, our results showed that HERPUD1 has regulatory roles on cell proliferation, migration, invasion and colony formation ability by affecting the cell cycle control, EMT and angiogenesis-related protein levels in MCF-7 breast cancer cells.

Study Limitations

In this study, the biological role of HERPUD1 on breast cancer was investigated *in vitro*. In order to better understand the role of HERPUD1 in the molecular pathogenesis of breast cancer are needed to test with further experimental studies and *in vivo* trials.

CONCLUSION

Our results suggest that targeting the HERPUD1 protein, which is an important component of the ER protein quality control mechanism, may be promising for selective treatment approaches to be developed for breast cancer.

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Figures and Figure Legends

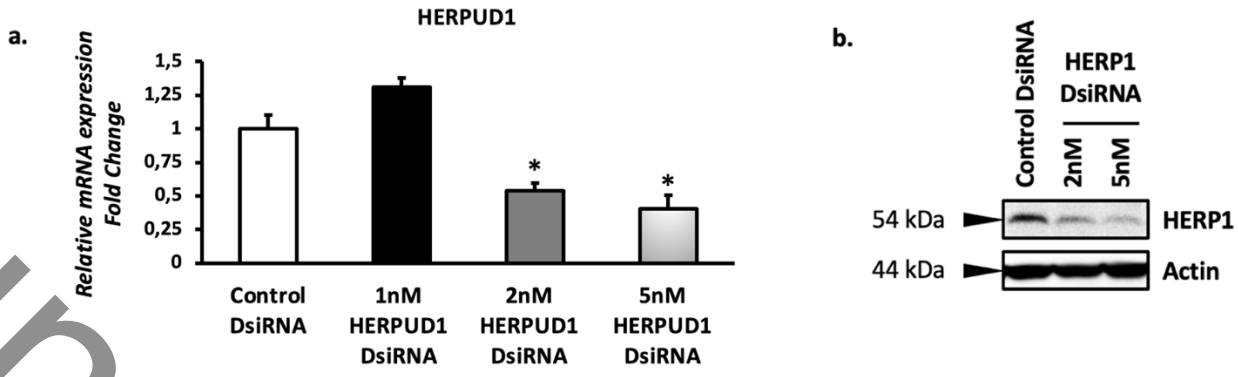

Figure 1: Optimization of effective HERPUD1 DisRNA concentration. (a) MCF-7 cells were transfected with HERPUD1 DsiRNA or control DsiRNA at 1, 2 and 5 nM concentrations for 72 hours, and then the changes in HERPUD1 mRNA expression were examined by qRT-PCR. GAPDH was used as a housekeeping gene and normalized relative quantification of the target genes was calculated according to GAPDH gene expression. Results are presented in terms of fold change compared to the control group (n=3) (*$p<0.05$). **(b)** Total protein isolation was performed in HERPUD1 DsiRNA or control DsiRNA transfected MCF-7 cells. HERPUD1 protein expression was analyzed by immunoblotting studies using HERPUD1-specific primary antibody. Beta-actin was used as a loading control.

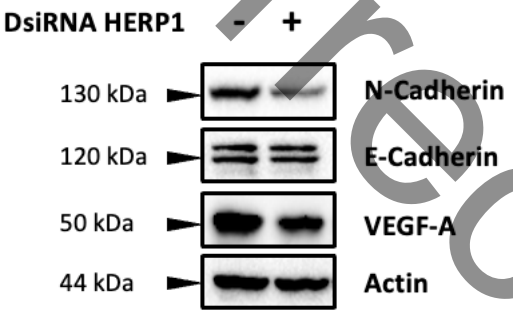

Figure 2: The effects of silencing of HERPUD1 on epithelial-mesenchymal transition and angiogenesis related protein levels. Changes in the level of N-cadherin, E-cadherin and VEGF-A protein expression were examined in MCF-7 cells transfected with 2 nM HERPUD1 DsiRNA and control DsiRNA by immunoblotting. Beta-Actin was used as a loading control.

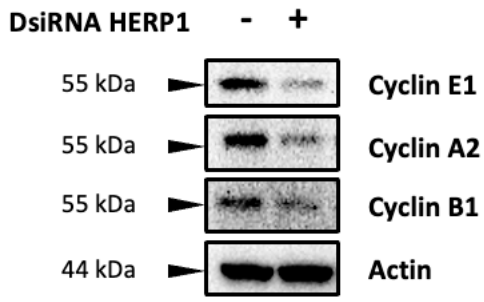

Figure 3: Investigation of the effects of the silencing of HERPUD1 expression on the levels of some cell cycle-related cyclin proteins in MCF-7 cells. Changes in the level of Cyclin A2, Cyclin B1 and Cyclin E1 protein expression were examined in MCF-7 cells transfected with 2 nM HERPUD1 DsiRNA and control DsiRNA by immunoblotting. Beta-Actin was used as a loading control.

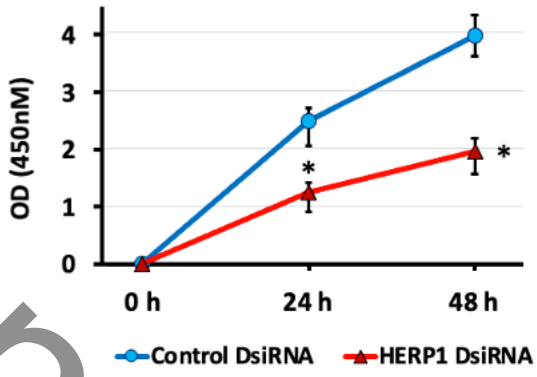

Figure 4: the effects of HERPUD1 silencing on proliferation. Proliferation changes in MCF-7 cells transfected with Control DsiRNA or 2 nM HERPUD1 DsiRNA were evaluated by WST-1 cell proliferation analysis as expressed in the method section. The spectrophotometric results (OD 450 nM) were presented in the graph (n=3) (*p<0.05).

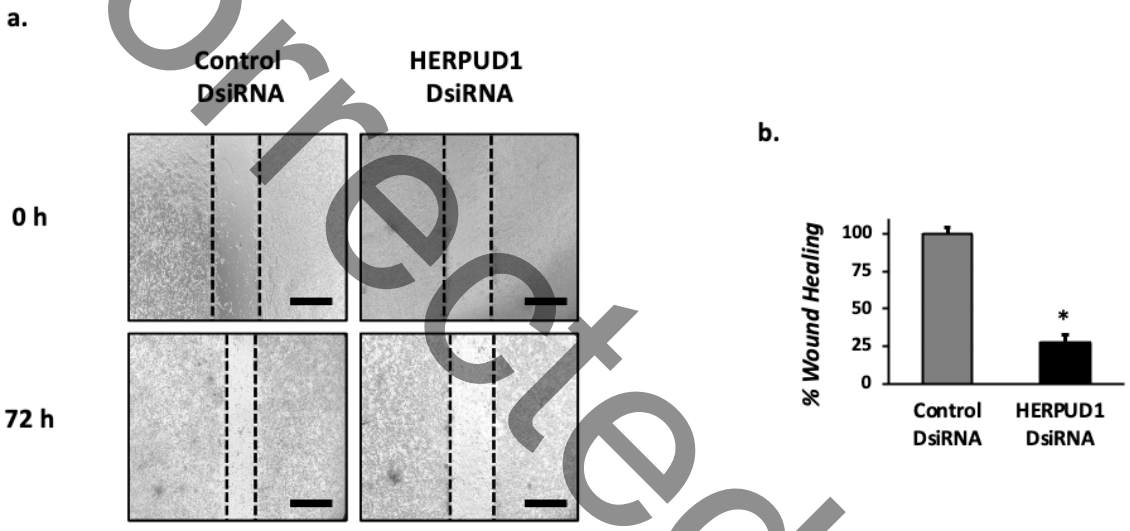

Figure 5: The effects of HERPUD1 silencing on wound-healing in MCF-7 cells. a) 0 and 72 hour of wound areas images (Scale: 50 µM). **b)** Graphical representation of wound closure rates. Closure rates were analyzed with ImageJ software and the results were presented in % folds changes (n=3) (*p<0.05).

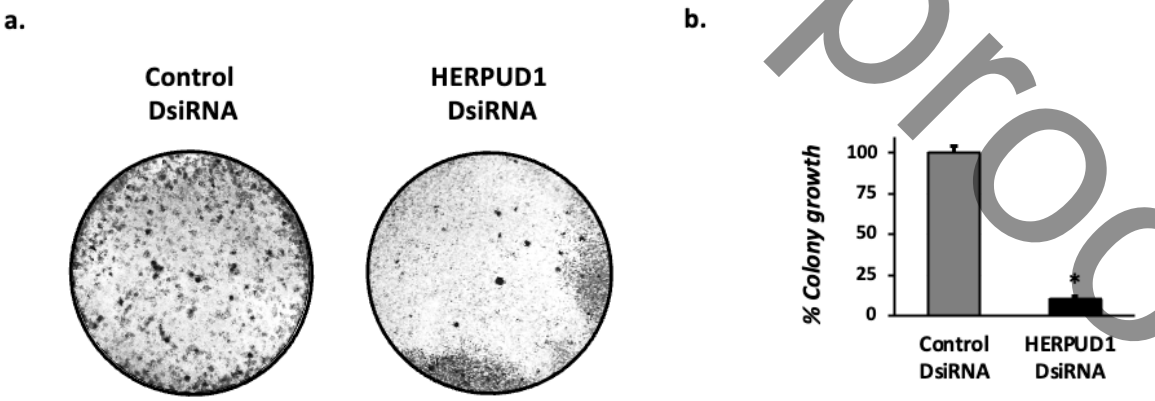

Figure 6: The effects of HERPUD1 silencing on colonial growth of MCF-7 cells. a) (a) Colonial growth of MCF-7 cells transfected with Control DsiRNA or HERPUD1 DsiRNA. **(b)** % Colonial growth changes were analyzed by ImageJ software and the results were presented in the graph (n=3) (*p<0.05).

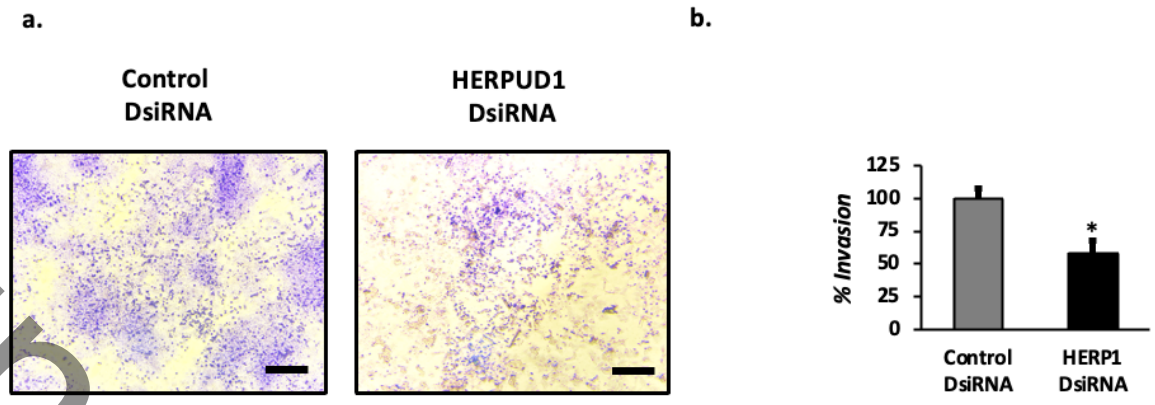

Figure 7: The effects of HERPUD1 silencing on an invasion of MCF-7 cells. MCF-7 cells were transfected with HERPUD1 DsiRNA or control DsiRNA at a concentration of 2 nM and invasion analysis was performed as described in the method section. **(a)** Staining of invaded cells on Transwell membrane with crystal violet solution (Scale: 50 µM). **(b)** Graphical representation of invading cells in terms of % fold change in the graph after quantification (n=3) *p<0.05.