INTRODUCTION

According to statistical studies conducted in the United States, breast cancer is the most frequently diagnosed cancer type and the second leading cause of cancer-related death in women.1 It is a hormonal regulation-dependent cancer type; because 70% of breast cancer cells contain estrogen receptors and/or progesterone receptors. Conservative surgery is the most commonly used method in treating breast cancer. Depending on

ABSTRACT

Objectives: Breast cancer is the most frequently diagnosed cancer type and the second leading cause of cancer-related death in women. Recent studies have highlighted the importance of the endoplasmic reticulum (ER) protein quality control mechanism for the survival of many cancers. It has also 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, which is an ER-resident protein quality mechanism. Today, the association of HERPUD1 with breast carcinogenesis is still not fully understood. Herein, we evaluated the possibility of HERPUD1 as a potential therapeutic target for breast cancer.

Materials and Methods: The effects of HERPUD1 silencing on epithelial-mesenchymal transition (EMT), angiogenesis, and cell cycle proteins were 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 human breast cancer cell line MCF-7. The statistical significance of the differences between the groups was determined by Student’s t-test.

Results: Our results displayed that suppressing HERPUD1 expression reduced the cell cycle-related protein levels, including cyclin A2, cyclin B1, and cyclin E1 in MCF-7 cells. Also, silencing of HERPUD1 remarkably decreased expression levels of EMT-related N-cadherin and angiogenesis marker vascular endothelial growth factor A. Moreover, we determined that cell proliferation, migration, invasion, and colony formation of MCF-7 cells were significantly limited by silencing of HERPUD1.

Conclusion: Present data suggest that HERPUD1 may be an effective target for biotechnological and pharmacological strategies to be developed to treat breast cancer.

Key words: Breast cancer, ERAD, HERPUD1, MCF-7
Numerous studies have reported that endoplasmic reticulum (ER) stress is highly activated in hematopoietic cancers (leukemia, lymphoma, and myeloma) and solid tumors, including stomach, colon, esophageal, lung, prostate, pancreas, liver, glioblastoma, and breast cancer as well. It has been determined that protein levels are associated with molecular chaperones, which are involved in protein quality control in ER and molecular mechanisms that mediate the removal of misfolded proteins to prevent proteotoxicity highly expressed in many cancer types. This process provides an opportunity for rapidly proliferating cancer cells to adapt against increased proteomic activity. Similarly, it is necessary for providing basal cellular needs and preventing apoptotic cell death signals.

ER is the largest organelle of the cell and functions as a center for crucial biological processes such as protein synthesis, transport, protein folding, lipid and steroid synthesis, carbohydrate metabolism, and Ca²⁺ storage. Protein folding is not a faultless process and it is known that one-third of newly synthesized proteins in cells are misfolded. Although proteins are properly folded, they frequently undergo conformational deterioration inside cells as they are constantly exposed to cellular stresses, such as heat and oxidative stress. This situation leads to release of hydrophobic residues in proteins that results in the formation of protein aggregates and cellular proteotoxicity. 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 ER and eliminating the misfolded proteins by directing them to the protein degradation process.

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

Homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) is a 54 kDa protein located in the ER membrane. It has been determined to play a role in the retrotranslocation step, which is expressed as the translocation of misfolded proteins from ER to the cytosol to direct 26S proteosome-mediated degradation for the elimination of misfolded proteins. To date, there is no identified enzymatic activity of HERPUD1. It has been shown that it regulates ERAD by interacting with proteins involved in the retrotranslocation, ubiquitination and degradation of misfolded proteins. HERPUD1 is one of the critical components involved in stabilizing the ERAD multiprotein complex and it effectively directs misfolded proteins to degradation and is thought to be one of the major components of ERAD. The interaction analyses showed that HERPUD1 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, adaptor subunit SEL1L, OS-9 acting as a lectin and Derlin1, which is proposed as a component of the channel complex. Furthermore, HERPUD1 expression is widely distributed in all tissues. In particular, high levels of its expression have been reported in tissues with advanced secretory ability, such as the pancreas. This feature of HERPUD1 suggests that it may play essential roles in tissues with high secretory properties, such as breast and prostate. Today, there is very limited data in the literature associating HERPUD1 with the carcinogenesis process. A study published in 2002 showed that androgens induce HERPUD1 gene expression in prostate cancer cells. Studies conducted with patient samples determined 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. Also, it was determined that apoptotic cell death was induced in prostate cells overexpressing HERPUD1. Another study exhibited that oxidative stress formation and cell adaptation genes were modulated by metformin administration and HERPUD1 was up-regulated among these genes in doxorubicin-resistant breast cancer cell lines.

In the present study, the effect of the DsiRNA-mediated suppression of HERPUD1 expression was investigated on tumorigenic properties of human breast adenocarcinoma cell line MCF-7. In this context, HERPUD1 expression was silenced by using DsiRNA and then its effects on proliferation, migration, invasion, and colony formation were evaluated in MCF-7 cells. Also, the effect of HERPUD1 on the cell cycle and epithelial-mesenchymal transition (EMT) was evaluated by immunoblotting. Our results showed that the suppression of HERPUD1 expression importantly decreased cell cycle-related protein levels in MCF-7 cells and reduced EMT. In addition, functional assay data indicated that silencing of HERPUD1 strongly limited all tested tumorigenic features of MCF-7 cells, including cell proliferation, migration, invasion, and colony formation abilities. Together, these results suggest that HERPUD1 may be an essential protein in the control of the tumorigenesis of breast cancer cells.

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) (1:3500), N-cadherin (22018-1-
AP) (1:1000), and E-cadherin (20874-1-AP) (1:1000) antibodies were obtained from Proteintech. Rabbit polyclonal cyclin E1 (#20808) (1:2500), cyclin A1 (#91500) (1:2500), and cyclin B1 (#12231) (1:2500) antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal vascular endothelial growth factor A (VEGF-A) (#E-AB-53277) (1:3000) antibody was provided from Elabscience. Mouse monoclonal beta-actin antibody (#A5316) (1:10000) was purchased from Sigma-Aldrich. HRP-conjugated goat anti-mouse (#31430) (1:5000) or goat anti-rabbit (#31460) (1:5000) IgG (H+L) was ordered from Thermo Scientific.

**Cell culture**

Human breast adenocarcinoma cell line MCF-7 (HTB-22®) was purchased from American Type Culture Collection (ATCC). The cells were propagated in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine under a humidified atmosphere of 5% CO₂ and 95% air at a constant temperature of 37 °C. The absence of mycoplasma contamination was routinely confirmed using the MycoAlert™ Mycoplasma Detection Kit (Lonza).

**siRNA transfection**

Negative control DsiRNA and HERP1 DsiRNA were ordered from Integrated DNA Technologies. DsiRNA transfection was performed by Xfect™ RNA transfection agent (Takara #631450) according to the manufacturer’s instructions. MCF-7 cells were seeded in 6 well cell culture dishes (3.5 x 10⁴ cells/well) and after 24 h, the transfection was applied. Cells were transfected with HERPUD1 DsiRNA or control DsiRNA at the indicated doses.

**RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using the NEB Monoarch™ miniprep total RNA isolation kit according to the manufacturer’s instructions (New England Biolabs). Quantification of the isolated RNA was determined using a micro-volume spectrophotometer (MySPEC, VWR). RNA (1 µg) was reverse transcribed by iScript™ cDNA synthesis kit following the manufacturer’s instructions (25 °C for 5 min, 46 °C for 20 min, and 95 °C for 5 min) (Bio-Rad). Gene expression of HERPUD1 was analyzed using iTaq Universal SYBR green reaction mix in a CFX96 RT-PCR instrument (Bio-Rad). The protocol steps were followed according to the manufacturer’s instructions. PCR primer sequences are available upon request. Fold change for the transcripts was normalized to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Relative quantification was analyzed by using 2^ΔΔCt method. In addition, melting-curve analysis was performed in the last step of qRT-PCR process to test specificity of the PCR reactions.

**Protein isolation and western blotting**

Protein isolation and immunoblotting studies were carried out as previously reported. MCF-7 cells were lysed with an ice-cold radiimmunoprecipitation assay buffer containing mammalian protease inhibitor cocktail; samples were centrifuged at 14,000 rpm for 30 min at 4 °C and the supernatant was stored for further studies. Total protein concentration was measured using bicinchoninic acid assay (Takara). Approximately 30 µg of protein samples was loaded on hand-cast SDS-PAGE gels and electrophoretically separated and then proteins were transferred to the polyvinylidene difluoride membrane. Following the transfer process, blocking, primary antibody incubation, washing, origin-specific horseradish peroxidase (HRP)-conjugated secondary antibody application, washing, and monitoring of the proteins were followed, respectively. Protein bands were monitored using clarity western enhanced chemiluminescence substrate solution in a ChemiDoc XRS+ system (Bio-Rad).

**The measurement of cell proliferation**

Cell proliferation was analyzed by WST-1-based cell proliferation assay according to the manufacturer’s instructions (Takara #MK400). Cells were seeded in a 96 well plate (5000 cells/wells). To measure cell proliferation, 10 µL/well premix WST-1 reactive was applied and cells were incubated for 2 h under conventional cell culture conditions. Absorbance reading at a wavelength of 450 nm was performed on a microplate reader (BioTek, Epoch 2). Measurements were taken at 24 and 48 h. The results were graphed to form proliferation curves.

**Colony formation assay**

Plate colony formation assay was performed as described before. MCF-7 cells were seeded in 6-well plates with 1000 cells/well and grown under conventional culture conditions for 72 h. At the end of the experiment, cells were washed twice with ice-cold 1X phosphate-buffered saline (PBS). Following this, colonies were fixed with alcohol and stained with 0.05% crystal violet solution (Sigma-Aldrich).

**Wound-healing assay**

Wound healing assay was achieved as mentioned before. MCF-7 cells were seeded in 12 well cell culture plates with 3.5 x 10⁵ cells/well and grown under conventional cell culture conditions. After 24 h, wounds were formed by using a yellow 200 µL tip and cells were washed twice with sterile 1X PBS to remove the cell residues. Following this, cells were grown in a culture medium for 72 h. Percentage of wound closure rates was analyzed with ImageJ software (http://imagej.nih.gov/ij/). The results were presented as mean ± standard deviation. The statistical significance of the differences between the groups was determined using the unpaired Student’s t-test. Differences were considered statistically significant when P < 0.05.
was determined using Student’s t-test with a minimum confidence interval of 95% using GraphPad Prism 7 software. A value of $p<0.05$ was considered significant.

**RESULTS**

**The determination of the optimum HERPUD1 DsiRNA concentration in MCF-7 cells**

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

**The investigation of the effects of HERPUD1 suppression on the epithelial-mesenchymal transformation in MCF-7 cells**

We analyzed the protein levels of *N*-cadherin and *E*-cadherin, considered markers associated with EMT, in HERPUD1-silenced MCF-7 cells by immunoblotting. Likewise, we tested the expression level of VEGF-A protein, which is associated with the angiogenesis process in cancer cells. Our findings revealed that silencing of HERPUD1 remarkably decreased *N*-cadherin protein levels, whereas *E*-cadherin levels were not affected compared with the control group (Figure 2). Moreover, the silencing of HERPUD1 strongly decreased the expression level of VEGF-A protein compared to the control group (Figure 2).

**The evaluation of the effects of silencing HERPUD1 on cell cycle-related proteins in MCF-7 cells**

Cyclin proteins are critical regulators for the uninterrupted progression of proteomic and genomic rearrangements throughout the cell cycle. These proteins are a family of proteins that activate specific cyclin-dependent kinases during phase transitions.\(^{23,24}\) To understand the effect of HERPUD1 protein on the control of the cell cycle in MCF-7 cells, we tested the levels of cyclin A2, cyclin B1 and cyclin E1 proteins in DsiRNA-mediated HERPUD1 suppressed MCF-7 cells by immunoblotting. Our results showed that HERPUD1 suppression remarkably decreased the levels of all tested cyclin proteins compared with the control group (Figure 3).

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

WST-1-based cell proliferation analysis was performed to evaluate the effects of HERPUD1 silencing on the proliferation of MCF-7 cells. Our results indicated that HERPUD1 suppression significantly reduced the proliferation of MCF-7 cells in a time-dependent manner compared with the control group (Figure 4).

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**Figure 1.** Optimization of effective HERPUD1 DsiRNA concentration. (a) MCF-7 cells were transfected with HERPUD1 DsiRNA or control DsiRNA at 1 nM, 2 nM and 5 nM concentrations for 72 h and then the mRNA expression levels of HERPUD1 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 the graph as a fold change compared to the control group (n = 3) (*$p<0.05$). (b) Total protein was isolated from HERPUD1 DsiRNA (2 nM, 5 nM) or control DsiRNA (5 nM) 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.** Investigation of the effects of DsiRNA-mediated HERPUD1 suppression on epithelial-mesenchymal transition and angiogenesis-related protein levels. The protein levels of *N*-cadherin, *E*-cadherin, and VEGF-A were examined in 2 nM HERPUD1 DsiRNA and control DsiRNA transfected MCF-7 cells by immunoblotting assay. Beta-actin was used as a loading control.

**Figure 3.** Evaluation of the effects of DsiRNA-mediated HERPUD1 suppression on cell cycle-related cyclins in MCF-7 cells. The protein levels of cyclin A2, cyclin B1 and cyclin E1 were examined in 2 nM HERPUD1 DsiRNA and control DsiRNA transfected MCF-7 cells by immunoblotting assay.
The investigation of the effects of silencing HERPUD1 on the wound healing ability of MCF-7 cells

Wound healing assay model is a relatively easy and cost-effective tool for evaluating the migration ability of cancer cells in vitro. Therefore, we used this assay system to test the effect of DsiRNA-mediated HERPUD1 suppression on the migration ability of MCF-7 cells. Our findings displayed that the migration of MCF-7 cells was significantly reduced by HERPUD1 suppression compared to control DsiRNA group (Figure 5a, b).

Testing the effects of silencing 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 Thus, to evaluate the relationship of HERPUD1 with tumorigenesis in breast cancer cells, we examined the effects of silencing HERPUD1 expression on colony formation ability in MCF-7 cells by using a 2D colony formation assay. Our results indicated that DsiRNA-mediated silencing of HERPUD1 expression significantly decreased the colony growth ability of MCF-7 cells compared with the control DsiRNA-transfected group (Figure 6a, b).

The evaluation of the effects of HERPUD1 suppression on the invasion capacity 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 or distant tissues and organs. It is an important factor leading to the spread of cancer cells and limiting their treatment efficacy.26 The effects of silencing HERPUD1 expression on invasion were evaluated by a Matrigel-coated Boyden-Chamber transwell assay in MCF-7 cells. Our data indicated that DsiRNA-mediated suppression of HERPUD1 expression significantly limited the invasion capability of MCF-7 cells compared with the control group (Figure 7a and b).

DISCUSSION

HERPUD1 has N- and C-terminal regions facing the cytoplasm and is localized in the ER membrane. Moreover, it can be induced through ER stress and homocysteine.14 HERPUD1 has been proposed as one of the significant components of ERAD, which acts as a structure-scaffolding protein in the ER membrane. It acts as a shuttle protein in the retrotranslocation step of ERAD and has no known enzyme activity. On the other hand, it is involved in the regulation of ERAD by interacting with proteins involved in ubiquitination and degradation processes in the retrotranslocation step of ERAD.15 Moreover, HERPUD1 controls the proteasomal degradation of the inositol 1,4,5-triphosphate receptor and ryanodine receptor proteins, which is a Ca²⁺ channel and has a critical role in Ca²⁺ homeostasis in this
way. HERPUD1 exhibits a comprehensive expression profile in all tissues and it has been reported to be expressed at high levels in tissues with high secretory ability, such as pancreas. This finding suggests that members of the ER protein quality control mechanism, an essential 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 breast and prostate.

Herein, we evaluate the effects of silencing HERPUD1 on the tumorigenic properties of MCF-7 breast cancer cells. A very limited data is available about the link between HERPUD1 and the carcinogenesis process in the literature. In studies conducted with samples from prostate cancer patients, it was reported that HERPUD1 expression was suppressed to a great extent in cancer group samples compared to healthy group samples. Another study showed that the administration of metformin to 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. Today, the functional effects of suppressing HERPUD1 expression in breast cancer cells are still unknown. Our data showed that DsiRNA-mediated silencing of HERPUD1 significantly limited the proliferative ability of MCF-7 cells compared with the control DsiRNA-treated group (Figure 4). In our microscopic examinations during this process, we did not observe any characteristics of cell death in HERPUD1-silenced MCF-7 cells.

Since EMT parameters are important in evaluating the tumorigenic capacity of cancer cells, we examined the E-cadherin and N-cadherin conversion and changes in VEGF-A, which is associated with angiogenesis. Our findings showed that the silencing of HERPUD1 markedly reduced N-cadherin levels, whereas E-cadherin levels were not affected compared to control DsiRNA-treated MCF-7 cells. Similar to N-cadherin results, VEGF-A levels were also importantly decreased in the HERPUD1-silenced group (Figure 2a). ER protein quality control mechanism tightly controls the levels of EMT proteins, tumor suppressors and oncogenic proteins. These results suggest that HERPUD1 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 proteins, we found that suppression of HERPUD1 caused a decrease in cyclin A2, B1 and E2 levels (Figure 2b). The levels of cyclin proteins are very important for the control of cell cycle phase transitions. Cyclin E1 regulates the progression of G1 phase to S phase. Some retrospective studies have presented an association between high cyclin E levels and an increased risk of death from breast cancer. 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. However, it does not always show a direct correlation with the degree of tumor aggressiveness. Cyclin B1 plays a regulatory role in the transition from the G2 phase to mitosis. It has been reported that cyclin B1 is overexpressed in primary breast, gastric and colorectal cancer cells. HERPUD1 suppression related decreased cyclin levels suggest that HERPUD1 may be directly related to regulating the steady-state levels of cell cycle phase transition proteins.

Advanced colonial growth and increased migration-invasion ability are considered hallmarks of the process of tumorigenesis. To test the possible role of HERPUD1 on the tumorigenic properties of breast cancer cells, we carried out wound healing, 2D colony formation, and matrigel-coated Boyden-Chamber invasion assays. Our data indicated that the colonial growth, migration, and invasion capacity of MCF-7 cells were significantly reduced by HERPUD1 suppression (Figures 4-7). Collectively, these functional data suggested that HERPUD1 has an essential role in regulating the tumorigenic features of MCF-7 cells.

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

CONCLUSION
Collectively, our results suggest that targeting the HERPUD1 protein, an essential component of the ER protein quality control mechanism, may be promising for developing selective treatment approaches for breast cancer.

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Authorship Contributions

Conflict of Interest: No conflict of interest was declared by the authors.

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