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Original Article

ERH is up-regulated in bladder cancer and regulates the proliferation and apoptosis of T24 bladder cancer cells

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Abstract: Bladder cancer is one of the malignant tumors that threaten human health. There is an urgent need for a biomarker or treatment target in the treatment of bladder cancer. In humans, enhancer of rudimentary homolog (ERH) is located on chromosome 14q24.1 and is expressed in almost all tissues. Analysis using StarBase v2.0 indicated that ERH expression is up-regulated in urothelial bladder cancer cells (211 cancer samples vs. 19 normal samples). StarBase v2.0 (http://starbase.sysu.edu.cn) starBase Pan-Cancer Analysis Platform is designed for deciphering Pan-Cancer Networks of lncRNAs, miRNAs, ceRNAs and RNA-binding proteins (RBPs) by mining clinical and expression profiles of 14 cancer types (>6000 samples) from The Cancer Genome Atlas (TCGA) Data Portal. Thus, we further explored the impact and mechanisms of ERH knockdown on the proliferation and survival of T24 human bladder cancer cells. The impact of ERH knockdown on cell proliferation, colony formation, and apoptosis was investigated in the T24 cell line using a lentiviral-mediated small interfering RNA (shRNA) strategy. The shRNA strategy efficiently reduced ERH expression, and ERH knockdown impaired cell proliferation, inhibited colony formation, and induced cell apoptosis in T24 human bladder cancer cells. Thus, our study provides evidence for the important role of ERH in the development and progression of human bladder cancer.

Keywords: ERH gene, bladder cancer, proliferation and apoptosis, gene knockdown

Introduction

Bladder cancer is one of the malignant tumors that threaten human health. If one did not receive an effective treatment, bladder cancer cells will transfer to a high-grade cancer, and the prognosis became worse. Therefore, to inhibit the tumor by chemical drugs may inhibit cancer cell proliferation. There is an urgent need for a biomarker or treatment target in the treatment of bladder cancer.

The enhancer of rudimentary homolog (ERH), a small protein of 104 amino acids, has been identified in plants, animals, and protists [1]. The mouse and human proteins have been synthesized and purified from *E. coli*, and the crystal structures have been determined [2, 3]. It has been suggested that ERH may be involved in many cellular functions, such as pyrimidine metabolism, cell cycle progression, and transcription control. Many studies have shown that ERH has a nuclear function. However, more accurate and convincing data are required to examine the relationship between this gene and human cancer cells. In the present study, the ERH gene was inhibited to explore the effect on the proliferation and apoptosis of a human bladder cancer cell line.

In the present study, TCGA (The Cancer Genome Atlas) data, including 230 bladder cancer sam-

ples, were downloaded from the StarBase v2.0 website to compare ERH mRNA expression between cancerousand normal tissues. Quantitative real-time PCR was also performed to quantify ERH mRNA expression in T24 and 5637 human bladder cancer cells. Furthermore, we used a lentiviral-based small interfering RNA (shRNA) strategy to knockdown ERH expression, and real-time PCR and Western blot analyses were subsequently performed to examine the knockdown efficiency. In addition, BrdU incorporation and Celigo assays were used to examine the cell proliferation of ERH-shRNA or scrambled-shRNA cell lines. A colony formation assay was used to assess the colony formation ability of ERH-shRNA T24 cells. Moreover, an annexin V-APC apoptosis assay was used to examine the apoptosis of cultured T24 cells following the addition of lentivirusexpressing ERH shRNA (shERH group) or scrambled shRNA (shCtrl group).

Materials and methods

Reagents

Fetal bovine serum (FBS, A11-102) was purchased from the Ausbian Pty Ltd. (155 Castlereagh St, Sydney New South Wales 2000). Dulbecco's minimum essential medium (DMEM, 10-013-CVR) and the Transwell kit (3422) were purchased from the Corning Company (One Riverfront PlazaCorning, NY 14831 USA). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, JT343] and trypsin (T04-58-50G) were purchased from the Bioengineering (Shanghai) Co., Ltd (No.151, Libing Rd., Zhangjiang Hi-Tech Park, Pudong, Shanghai, China). D-Hanks was obtained from the Shanghai Genechem Technology Co., Ltd. (332 Edison Road, Zhangjiang Hi - Tech Park, Pudong New Area, Shanghai, China), and the Sigma Cable Company (Pte) Ltd. (19 Benoi Road, Sigma High Tech Complex, Jurong Town, Singapore) provided Giemsa Staining Solution (32884). The Shanghai First Chemical Reagent Co., Ltd. (1317 Jianchuan Road, Minhang District, Shanghai, China) supplied DMSO (dimethyl sulfoxide, 130701). Anti-GAPDH (sc-32233) and goat anti-mouse IgG secondary antibodies (sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (10410 Finnell Street, Dallas, Texas 75220, U.S.A.). The BCA Protein Assay kit (P0010S) and RIPA lysis buffer (strong) (P0013B/) were obtained from the Beyotime Institute of Biotechnology (No.123, Rongle East Road, Songjiang District, Shanghai, China). RIPA lysis buffer (WB-0071) and NP-40 lysis buffer (P0013F) were purchased from the Shanghai Dingguo Biotechnology Co. Ltd. (Anshun Road 89, Lane on the 7th River Building, Room 1202, Changning District, Shanghai, China). Prestained protein markers (00161543) and the ECL-PLUS Kit (M3121/1859022) were purchased from the Thermo Fisher Scientific Co. Ltd. (Thermo Fisher Scientific, 168 Third Avenue, Waltham, MA USA 02451). Medical X-ray film (038401501) was obtained from the Carestream Company (150 Verona Street, Rochester, NY, USA). X-ray film developer powder and fixing powder (P61-04-1) were purchased from the Shanghai Guanlong Photographic Material Factory (No.221, Jinling East Road, Shanghai, China).

Cell culture

The bladder cancer cell lines T24 and 5637 were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences at the Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium containing 10% FBS, streptomycin, and penicillin at 37°C in an incubator with 5% CO_2.

Data sources and bioinformatics

The ERH expression level in bladder cancer tissues and corresponding non-tumor tissues in The Cancer Genome Atlas (TCGA) database were analyzed by StarBase database. StarBase v2.0 (http://starbase.sysu.edu.cn) starBase Pan-Cancer Analysis Platform is designed for deciphering Pan-Cancer Networks of lncRNAs, miRNAs, ceRNAs and RNA-binding proteins (RBPs) by mining clinical and expression profiles of 14 cancer types (>6000 samples) from The Cancer Genome Atlas (TCGA) Data Portal.

ERH shRNA design and lentivirus construction

To inhibit ERH expression, shRNA specifically targeting ERH (target sequence: CTGGTTTACCGAGCTGATA) was designed and packed into a lentivirus vector. A scrambled shRNA was used as a negative control (shCtrl) using the following sequence: TTCTCCGAACGTGTCACGT. T24 human bladder cancer cells were used to examine the knockdown efficiency at the mRNA level, and 293T cells were used to examine the

Table 1. The ΔCt of the human bladder cancer T24 cell line was 4.57 ± 0.17, and the ΔCt of the human bladder cancer 5637 cell line was 2.61 ± 0.04. The results of real-time PCR showed that ERH was positively expressed in both T24 and 5637 human bladder cancer cells

Tumor cell line	ΔCt
T24 cell line	4.57 ± 0.17
5637 cell line	2.61 ± 0.04

knockdown efficiency at the protein level. The cells were plated onto 6-well plates and subsequently infected with lentivirus expressing ERH shRNA. The cells were cultured at 37°C in an incubator with 5% CO_2 until 30% confluence. Next, the cells were harvested, and total RNA and protein were extracted to determine the knockdown efficiency using real-time PCR and Western Blot assays. When the infection efficiency was greater than 70%, the cellswere usedfor the downstream experiment.

Quantitative real-time PCR was performed to quantify the ERH mRNA expression in human bladder cancer T24 and 5637 cells and to examine the knockdown efficiency in human bladder cancer T24 cells. Total RNA was extracted and purified using Trizol reagent according to the manufacturer's instructions. Reverse transcription was performed to obtain cDNA using M-MLV reverse transcriptase. ERH mRNA expression was measured using a quantitative real-time PCR assay with SYBR master mixon an MX3000p real-time PCR machine (Agilent).

Western blotting was performed to quantify the expression of ERH protein in T24 cells. The cells were washed twice using cold PBS after 48 h of infection. The BCA approach was used to detect the protein density. Equal amount of total proteins (30-50 μg) were resolved using 10% Bis-Tris gradient SDS-PAGE under reducing conditions and were then transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% skim milk for 1 h. The membranes were incubated with primary antibodies or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody at room temperature for 2 h. The membranes were washed 4 times with TBST (8 min each) and incubated with fluorescein-linked secondary antibodies at room temperature for 1.5 h. Next, the membranes were washed another 4 times with TBST (8 min each), and signal detection was performed using ECL and X-ray film.

Cell proliferation

The BrdU incorporation assay was used to examine the cell proliferation of cultured T24 cells following the addition of lentivirus expressing ERH shRNA (shERH group) or scrambled shRNA (shCtrl group). After the cells were plated onto 96-well plates and incubateduntil they reached a suitable density, BrdU reagents diluted 1:100 were subsequently added to each well (10 μL/well). Cell proliferation was analyzed using aBrdU incorporation assay using a BrdU Cell Proliferation ELISA kit (Cat. No. 11647229001, Roche Applied Science) according to the manufacturer'sinstructions. The BrdU density was 450 nm using Biotek Elx800, and the experiments were performed in triplicate.

The Celigo system was used to compare cell proliferation between 2 groups in the same microscope field. Lentivirus expressing ERH shRNA (shERH group) or scrambled shRNA (shCtrl group) was added to cultured T24 cells; the cells were subsequently incubated for another 72 h, and the cell suspensions were obtained. The cells were plated onto 96-well plates at a density of 2,000 cells/well. After culturing at 37°C in an incubator with 5% CO_2 for another 24 h, the cell images were captured using Celigo (Nexcelom) once a day for 5 days. The number of cells per well was also quantified using the same machine, and cell growth curves were created for each condition.

Colony forming cell assay

After infection with lentivirus expressing ERH shRNA or scrambled shRNA, T24 cells were cultured for another 72 h and harvested in the logarithmic phase. The cells were subsequently counted and plated in triplicate onto 6-well plates at a density of 700 cells per well. After incubating in a 5% CO_2 incubator at 37°C for 7 days, the cells were washed with PBS and subsequently fixed with paraformaldehyde for 30 to 60 min. The cell colonieswere stained with 500 μL of Giemsa for 20 min at room temperature. Images of the cell plates were captured, and the colonieswere analyzed.

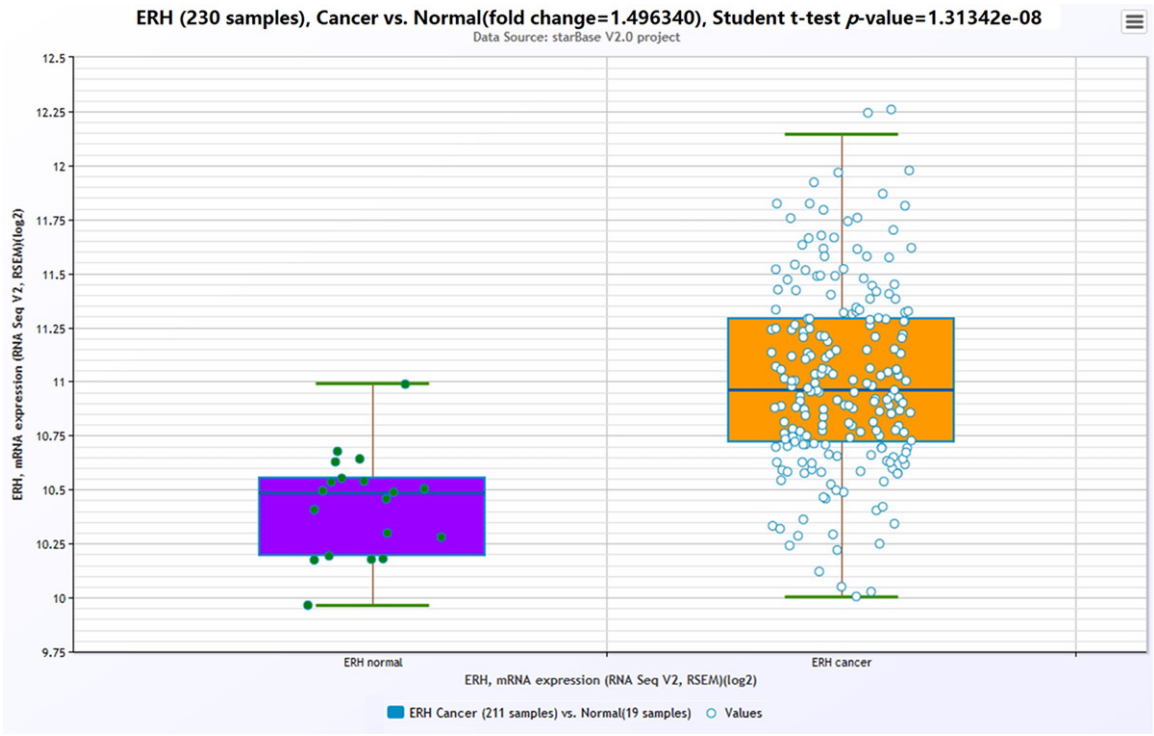

Figure 1. Enrichment analysis indicated that ERH expression was up-regulated in urothelial bladder cancer cells (Source: HGNC Symbol, http://starbase.sysu.edu.cn/viewGeneInfo.php?table=miRNAClipIntersectTargets&database=hg19&name=ERH).

Annexin V-APC apoptosis assay

Initially, T24 cells were infected with lentivirus expressing ERH shRNA or scrambled shRNA. The cells were harvested after incubation for another 120 h and subsequently washed with PBS buffer and resuspended using staining buffer at a density of 1×10^5 - 1×10^6/mL. Subsequently, 5 μL Annexin V-APC was added to 100 μL of cell suspension and incubated at room temperature for 10-15 min. The signals were detected using a FACS Calibur (Millipore).

Statistical analysis

Statistical analyses were performed using SAS 8.02 (SAS Institute Inc., Cary, NC, USA). Significant differences of continuous data (mean ± SD) were estimated using the Student's t test. Including ERH expression at the mRNA and protein levels, Clonogenic ability and cell apoptosis compared between T24 and ERH knockdown T24 human bladder cancer cell line. Comparison of the cell proliferation was analyzed using one-way analysis of variance (ANOVA). A p-value <0.05 was defined as statistically significant.

Results

ERH was expressed in the T24 and 5637 human bladder cancer cell lines and was up-regulated in human bladder cancer cells

We evaluated ERH expression using real-time PCR in the T24 and 5637 human bladder cancer cell lines, and the results showed that ERH was expressed in the two cell lines. The ΔCt of T24 cells was 4.57 ± 0.17, and the ΔCt of 5637 cells was 2.61 ± 0.04 (**Table 1**). The results of real-time PCR showed that ERH was expressed in both T24 and 5637 cells. Analysis using the StarBase v2.0 indicated that ERH expression was up-regulated in urothelial bladder cancer cells (**Figure 1**, 211 cancer samples vs. 19 normal samples. Fold-change =1.49340, Student's t-test p-value =$1.3134e^{-08}$).

ERH expression was efficiently inhibited after-lentiviral-based shRNA in T24 human cancer cells at the mRNA level and at the protein level

A lentiviral-based shRNA strategy was used to knockdown ERH in human bladder cancer T24 cells. Lentivirus expressing shRNA specifically

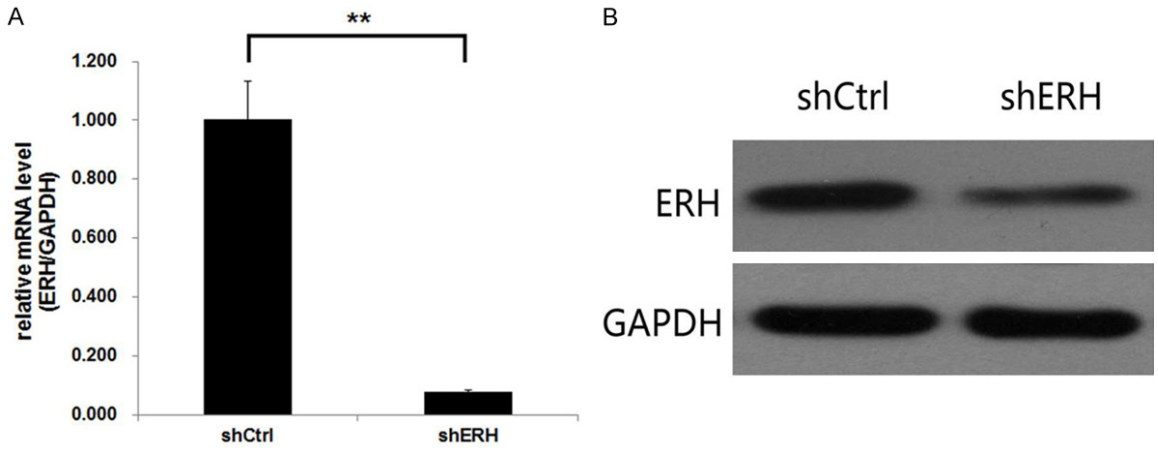

Figure 2. A. The mRNA level following ERH knockdown using lentiviral-based shRNA was detected using quantitative real-time PCR in T24 human bladder cancer cells. ERH expression at the mRNA level was reduced approximately 92.1% in T24 cells ($p<0.01$). B. The protein level following ERH knockdown using lentiviral-based shRNA was detected using Western Blotting in T24 cells. ERH expression at the protein level was reduced, while GAPDH was equally expressed between the shCtrl and shERH groups.

targeting human ERH (ERH-shRNA, shERH group) or a scrambled sequence (ScrshRNA, shCtrl group) was initially generated. Subsequently, T24 cells were infected with lentivirus expressing ERH-shRNA or ScrshRNA to evaluate the knockdown efficiency. T24 cells were harvested, and total RNAs and protein were collected. Quantitative real-time PCR and Western Blot were performed to determine ERH expression at the mRNA and protein levels, respectively. ERH expression at the mRNA level was reduced approximately 92.1% in T24 cells. ERH expression at the protein level was also reduced in T24 cells (**Figure 2**). These results showed that the lentiviral-based shRNA strategy could effectively and efficiently inhibit ERH expression at both the mRNA and protein levels.

Cell proliferation was impaired after ERH knockdown in the T24 human bladder cancer cell line

Sustained proliferation is one of the important hallmarks of cancer cells [4]. Thus, weexamined the impact on ERH knockdown on cell proliferation ofthe T24 human bladder cancer cell line.

A BrdU incorporation assay was performed to examine the proliferation of cultured T24 cells following the addition of lentivirus expressing ERH shRNA (shERH group) or scrambled shRNA (shCtrl group). Cell proliferation was evaluated based on the BrdU incorporation ratio, which showed that compared to shCtrl, ERH-shRNA significantly inhibited cell proliferation ($p<0.05$). Celigo analysis was performed to compare the cell proliferation between 2 groups in the same microscope field. The results were consistent with the BrdU assay results, showing that ERH-shRNA significantly inhibited cell proliferation compared to shCtrl (**Figure 3**).

The clonogenic ability of T24 human bladder cancer cells was inhibited by using ERH-shRNA

Replicative immortality is another crucial hallmark of cancers, and it manifests as an unlimited replicative potential and a highly clonogenic ability [5]. The clonogenic ability of cells infected with lentivirus expressing ERH or shCtrl was evaluated and compared using a colony formation assay (**Figure 4A** and **4B**). The results showed that the clonogenic ability was significantly inhibited after ERH knockdown.

Cell apoptosis was promoted through ERH knockdown in human bladder cancer T24 cells

An annexin V-APC apoptosis assay was performed to examine the cell apoptosis of cultured T24 cells following the addition of lentivirus expressing ERH shRNA (shERH group) or scrambled shRNA (shCtrl group). The results showed that ERH-shRNA significantly promoted

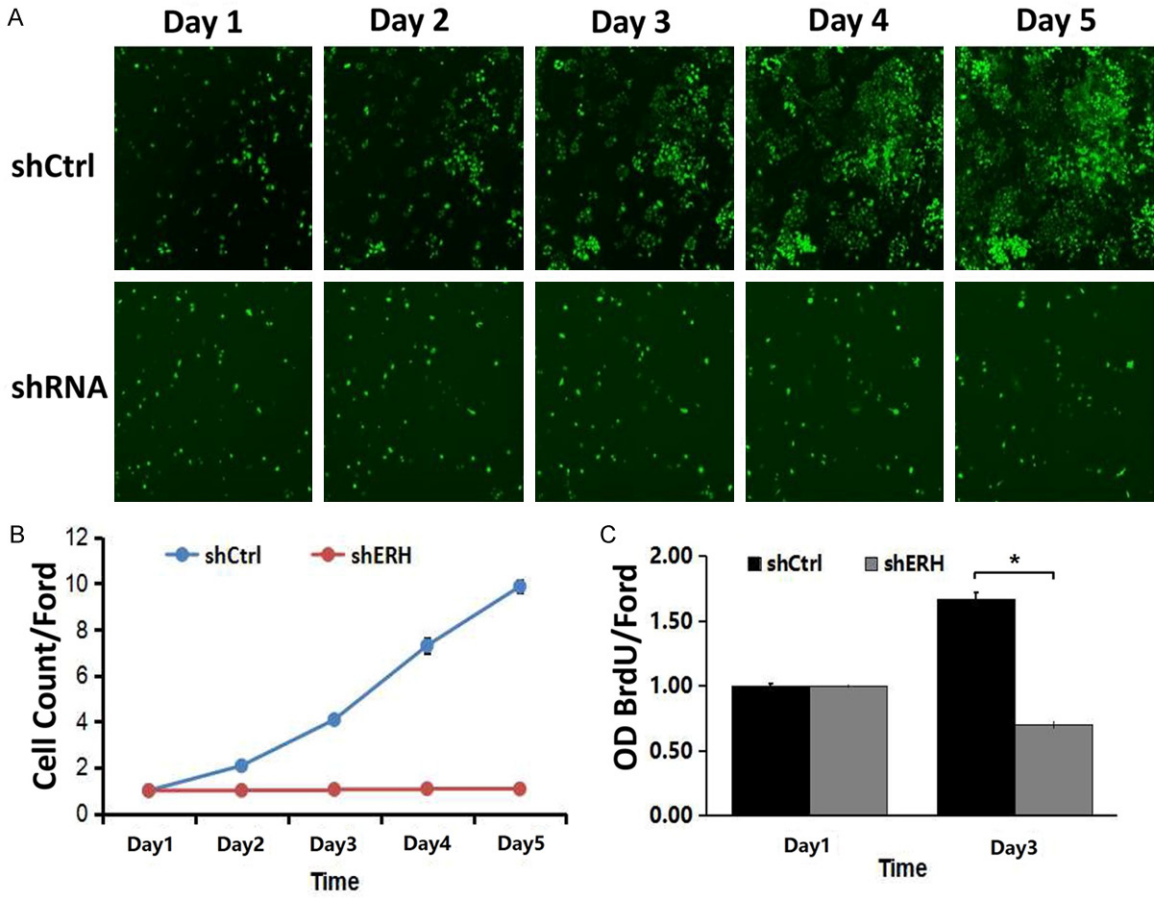

Figure 3. Cell proliferation after ERH knockdown in T24 human bladder cancer cellswas detected using Celigo analysis and the BrdU incorporation assay. A. Representative images of human bladder cancer cells infected with lentivirus expressing Scr-shRNA (top, shCtrl group) or ERH-shRNA (bottom, shERH group) at different time points post-infection. Images of T24 cells infected with lentivirus expressing Scr-shRNA or ERH-shRNA are shown. B. Proliferation of T24 cells was analyzed using Celigo for cell number quantification. The cells were infected with lentivirus expressing either ERH-shRNA or Scr-shRNA and cultured for 5 days. The cell number was quantified dailyusing Celigo. Proliferation is shown as the fold-change in cell number, and the fold-changeson other dayswas normalized to day 1. The results represent the mean ± SD of five separate experiments (*p<0.05). C. The proliferation of T24 cells expressing either Scr-shRNA or ERH-shRNA was analyzed via BrdU incorporation assays. The BrdU incorporation ratio is represented as the fold-change of absorbance at 450 nm (OD BrdU/fold). The OD BrdU/fold was quantified daily for 3 days. Day 3 refers to the BrdU incorporation ratio in cells 72 h after lentivirus infection. Data are presented as the mean ± SD of 4 independent experiments (*p<0.05).

cell apoptosis compared to shCtrl (p<0.05, **Figure 4C** and **4D**).

Discussion

ERH is a small protein of 104 amino acids [1] that was discovered in *Drosophila* more than 20 years ago. ERH is a highly conserved protein found in most eukaryotes [2]. ERH is essential for the suppression of meiotic mRNAs during normal cell growth. Because this rudimentary gene encodes a CAD-like enzyme in the pyrimidine biosynthetic pathway, it may have an effect on the regulation of pyrimidine metabolism. The ERH gene in humans is located on chromosome 14q24.1 and is expressed in almost all tissues [6]. ERH does not contain a known functional domain. The ERH protein contains three α-helices and a β-sheet folded into a novel structure comprising a single domain, with the β-sheet comprising four antiparallel β-strands [7, 8]. Purified ERH protein forms homodimers in solution, suggesting that the ERH protein may function as a dimer in cells [7]. A recent study suggested [9] that ERH protein and the RNA-binding protein Mm 1 form a complex named EMC, which promotes meiotic mRNA

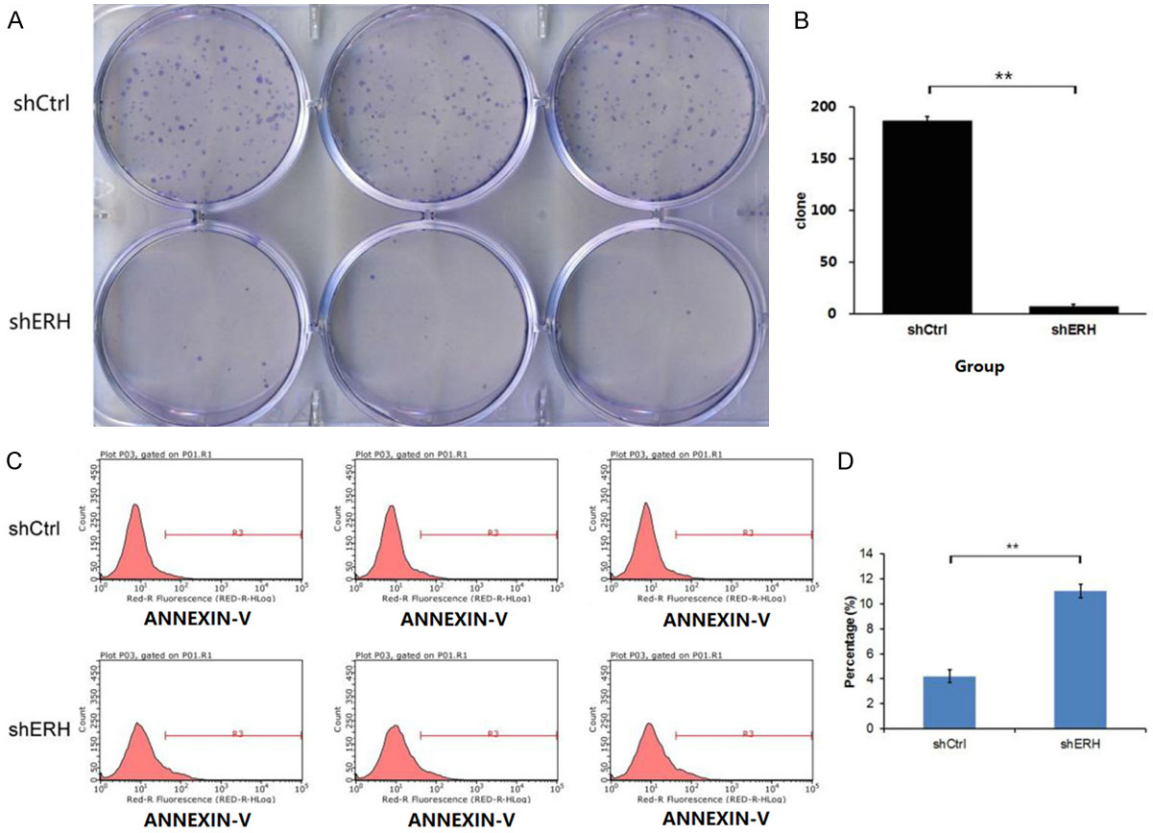

Figure 4. A colony formation assay shows impaired clonogenic ability in T24 bladder cancer cells through ERH knockdown based on a colony formation assay. Cell cycle arrest and apoptosis in T24 human bladder cancer cells following ERH knockdown were measured using theannexin V-APC apoptosis assay. A. Representative images of the colony formation of T24 cells infected with lentivirus expressing either Scr-shRNA (top, shCtrl group) or ERH-shRNA (bottom, shERH) in a 6-well plates with Giemsa staining performed on the 7th day. B. Quantification of the number of clones formed by T24 cells infected with lentivirus expressing either Scr-shRNA (left, group shCtrl) or ERH-shRNA (right, shERH group) on the 7th day. The results represent the mean ± SD of three separate experiments (**p<0.01). C. The cell cycle distribution of cells infected with lentivirus expressing Scr-shRNA (top, shCtrl group) or ERH-shRNA (bottom, shERH group). D. Quantification of cell apoptosis in cells infected with lentivirus expressing Scr-shRNA (left, shCtrl group) or ERH-shRNA (right, shERH group). Data are presented as the mean ± SD of the cell percentage showing apoptosis from three separate experiments (**p<0.01).

decay and facultative heterochromatin assembly.

ERH may also play a role as a transcriptional regulator and elongator. Pogge von et al. [10] showed that *Xenopus* ERH interacts with the transcriptional co-activator of HNF1/pterin-4a-carbinoamine dehydratase (DCoH/PCD). Kwat et al. [11] showed that ERH interacts with the transcription factor SPT5, which regulates RNA polymerase II elongation.

ERH has also been proposed to function in the cell cycle. Lukasik et al. [12] showed that ERH is coexpressed with Ciz1, a nuclear zinc-finger protein, in HeLa cells. These authorsobserved that ERH could block the binding of $p21^{Cip1/Waf1}$, an important inhibitor of cyclin E-cyclin-dependent kinase 2 (CDK2), through Ciz1 in the cell. Smyk et al. [6] showed that ERH is a molecular partner of PDIP46/SKAR, a protein interacting with DNA polymerase and S6K1 and regulating cell growth. It has further been suggested that ERH interacts with the spliceosome protein SNRPD3, which is required for splicing of the mRNA of the mitotic motor proteinCENP-E in HeLa cells [13]. ERH also interacts with protein-arginine methyltransferase 1 (PRMT1), which is associated with mRNA splicing [14].

Although the ERH gene might not be a target for point mutations or deletions/amplifications in

human carcinoma [15], this gene is more abundantly expressed in human breast cancer cells than in samples of healthy tissue [16]. A recent study [13] showed that ERH knockdown down-regulated gene expression as a consequence of mRNA splicing defects in DLD-1 human colorectal cancer cells. It was also demonstrated that [17] ERH interacts with H19 as a tumor growth inhibitor in Wilms' tumor cell lines. Fujimura et al. [18] demonstrated that ERH is essential for the progression of the mitotic phase of the cell cycle by establishing the alignment of mitotic chromosomes in HeLa cells. Recent data [19] further suggest that ERH is an important survival factor in certain pancreatic, breast, and ovarian cancer cell lines. A recent study [20] showed that ERH targetsthe Chk1 inhibitor to regulate the splicing of the DNA damage response protein ATR (ataxia telangiectasia and Rad3-related kinase) in hepatocellular carcinoma cells. These authors further showed that the down-regulation of ERH through siRNAs decreases the splicing efficiency of ATR mRNA. Further studies showed that ERH was important for ATR signaling but was not observed at sites of replication or DNA damage.

Without effective therapy, bladder cancer, one of the leading causes of cancer-related death worldwide, may progress from low to high grade. Cell proliferation induced through chemicals is a key factor in cancer progression [21]. Currently, there are no studies investigating the proliferative effects of ERH inhibition on human bladder cancer cells.

The results of the present study showed that the ERH gene was abundantly expressed in human bladder cancer cells. We successfully suppressed the ERH gene in bladder cancer cell lines using a lentivirus, andthis lentiviral-based shRNA strategy could efficiently inhibit ERH expression at both the mRNA and protein levels.

Studies concerning the relationship between ERH, human bladder cancer cells proliferation and apoptosis remain limited. However, the results of previous studies are consistent with the observations of the present study, revealing that ERH suppression in human bladder cancer cells leads to cell cycle arrest and promotes cell apoptosis. Although the phase of cell cycle arrest in bladder cancer cells and the molecular mechanisms underlying ERH-mediated cancer progression have not been fully explored, further studies into the detailed molecular mechanisms will expand the current knowledge of ERH in cell proliferation and apoptosis in human bladder cancer cells.

In the present study, the ERH gene was inhibited to explore its effect on the proliferation and apoptosis of human bladder cancer cell lines. The results showed that ERH knockdown inhibited proliferation and promoted apoptosis in T24 human bladder cancer cells.

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