Article

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HEATR1 Promotes Glioblastoma Cell Proliferation, Migration, and Invasion Through Regulation of Nrf2

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Background: HEAT repeat-containing protein 1 (HEATR1) has been reported to play key roles in tumor progression, but its expression patterns and functional significance in glioblastoma (GBM), particularly in relation to the nuclear factor erythroid 2-related factor 2 (Nrf2) regulatory pathway, remain poorly understood. Therefore, this study aimed to investigate the expression profile of HEATR1 in GBM and to elucidate its functional role and underlying molecular mechanisms, with a specific focus on its regulatory relationship with Nrf2.

Methods: Bioinformatics analyses using GEPIA2, cBioPortal, and the Human Protein Atlas were employed to evaluate HEATR1 expression and its correlation with Nrf2 in GBM. Functional assays, including shRNA-mediated gene silencing, Nrf2 overexpression, Cell Counting Kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), wound healing, and Transwell invasion, were performed in U87 and U251 cell lines. Quantitative real-time PCR (qRT-PCR) was employed to assess mRNA levels, and protein abundance was determined using Western blot analysis.

Results: Knockdown-mediated silencing of HEATR1 expression led to a pronounced reduction in the proliferative, migratory, and invasive abilities of GBM cells. From a mechanistic perspective, HEATR1 functioned as a positive regulator of Nrf2, enhancing its expression at both transcriptional and translational levels; however, the regulatory relationship appeared to be unidirectional, since alterations in Nrf2 expression did not exert feedback effects on HEATR1 expression. Experimental rescue analysis revealed that elevating Nrf2 expression could partially mitigate the inhibitory consequences of HEATR1 depletion on the proliferative, migratory, and invasive properties of GBM cells.

Conclusion: HEATR1 promotes glioblastoma progression by upregulating Nrf2, thereby enhancing cellular proliferation, migration, and invasion. The HEATR1–Nrf2 axis may represent a promising therapeutic target in GBM.

Keywords: glioblastoma; HEATR1; Nrf2; tumor progression

Introduction

Glioblastoma multiforme (GBM) is the most lethal central nervous system (CNS) tumor. In 2020, approximately 300,000 new cases of brain and central nervous system cancers were diagnosed globally, contributing to an estimated 250,000 deaths [1]. Notably, GBM constitutes nearly half of all CNS malignancies, underscoring its significant clinical burden.

HEAT repeat-containing protein 1 (HEATR1) is a high-molecular-weight protein composed of a polypeptide chain containing 2144 amino acid residues, and its most notable structural hallmark lies in the HEAT repeat motif located within the C-terminal region. This structural domain exhibits evolutionary conservation and can be identified in a variety of important proteins, such as protein phosphatase 2A (PP2A), the elongation factor-3, as well as the huntingtin protein [2], which has been associated with essential cellular functions. Accumulating research has indicated that HEATR1 exerts diverse and complex regulatory functions in the biological behavior of gliomas. On the one hand, HEATR1 has been recognized as an antigen closely linked to glioma, which has the ability to stimulate the generation of functional cytotoxic T lymphocytes in patients suffering from this tumor, indicating its potential involvement in tumor immunogenicity [3]. On the other hand, it has been shown that HEATR1 cooperates with MYC to drive ribosome biogenesis, thereby promoting glioma cell proliferation and tumor progression [4]. These findings suggest that HEATR1 may exert context-dependent functions in glioma, potentially participating in both immune surveillance and oncogenesis. Given the complexity of its biological activities, clarifying the underlying molecular pathways through which HEATR1 influences the progression of glioma, particularly its interaction with oncogenic signaling pathways, is of considerable research interest and may provide novel insights into glioma pathogenesis.

Previous research has documented that HEATR1 regulates the signaling pathway of nuclear factor erythroid 2-related factor 2 (Nrf2), thereby influencing both tumor cell proliferation and chemoresistance in pancreatic cancer, particularly under gemcitabine treatment [5]. Nrf2 functions as a pivotal transcription factor that maintains cellular redox balance, primarily by regulating a wide array of genes responsible for the production of antioxidant enzymes and cytoprotective proteins, thereby safeguarding cells from oxidative or electrophilic stress [6,7]. Dysregulated activation of the Nrf2 signaling cascade has been documented across multiple solid malignancies, such as gliomas, where it is strongly correlated with increased cellular proliferation, invasive capacity, and reduced sensitivity to chemotherapeutic agents [8–10]. Considering the documented association between HEATR1 and the Nrf2 signaling pathway, together with the well-established role of Nrf2 in glioma development and progression, this relationship warrants further in-depth investigation. It is of considerable interest to investigate whether HEATR1 contributes to glioma progression through modulation of the Nrf2 axis. Therefore, exploring how HEATR1 functionally interacts with the Nrf2 signaling cascade in glioma may reveal novel molecular mechanisms underlying tumor progression and provide new opportunities for the development of more precise therapeutic strategies.

Materials and Methods

Bioinformatics Analysis

HEATR1 and Nrf2 expression in GBM tissues, as well as in normal brain samples, was analyzed using the GEPIA database (http://gepia.cancer-pku.cn/), which integrates data from both the TCGA and GTEx projects. Differences in transcript levels were visualized using the Expression module, with significance set at $p < 0.05$.

HEATR1 protein expression in glioma and normal brain tissues was assessed using immunohistochemical images retrieved from the Human Protein Atlas (HPA, https://www.proteinatlas.org/). Differences in staining intensity and spatial distribution were analyzed to evaluate variations in protein-level expression.

In addition, to explore the potential regulatory relationship between HEATR1 and Nrf2, correlation analysis was performed using the cBioPortal for Cancer Genomics (https://www.cbioportal.org/), based on the TCGA-GBM cohort. The co-expression patterns of HEATR1 and Nrf2 in GBM samples were assessed using the “Co-expression” module.

Cell Culture

Normal human astrocytes (NHA, #CP-H122), along with GBM cell lines A172 (#CL-0012), U87 (#CL-0238), and U251 (#CL-0237), were acquired from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The identity of every cell line was validated by short tandem repeat (STR) profiling, and subsequent testing confirmed that none were contaminated with mycoplasma.

NHA cells were cultured in Astrocyte Medium (#CM-H122, Procell, Wuhan, China) enriched with 2% fetal bovine serum (FBS), 1% astrocyte growth supplement, and 1% penicillin-streptomycin solution. Meanwhile, A172, U87, and U251 cell lines were maintained in high-glucose Dulbecco’s Modified Eagle Medium (DMEM, #C11995500BT, Gibco, Grand Island, NY, USA), supplemented with 10% FBS (#10099141C, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (#15140122, Gibco, Grand Island, NY, USA). All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell Transfection

Lentiviral shRNAs targeting HEATR1 (shH) and Nrf2 (shN), along with a non-targeting control (shCtrl), were obtained from GenePharma (Shanghai, China). The targeting sequences as follows: shH1: 5′-TGAACAAGTCCGAATAGAA-3′, shH2: 5′-GCTGAACAAGTCCGAATAGAA-3′; shN1: 5′-GCAGTTCAATGAAGCTCAACT-3′, shN2: 5′-GATGAAGAGACAGGTGAATTT-3′; shCtrl: 5′-TTCTCCGAACGTGTCACGT-3′. These provided sequences represent the targeting regions. Nrf2 overexpression was achieved by transfecting cells with pcDNA3.1(+) (#V79020, Invitrogen, Carlsbad, CA, USA) carrying full-length Nrf2 cDNA (#puno1-hnfe2l2, Invivogen, Carlsbad, CA, USA), with empty vector as control.

U87 and U251 cells at 80% confluency were infected with lentiviral particles (MOI = 20) in the presence of 8 µg/mL polybrene (#H9268, Sigma-Aldrich, St. Louis, MO, USA) and cultured for 24 h. After infection, the medium was replaced with fresh complete medium, and cells were further cultured for an additional 48 h before selection. Stable cell lines were selected with puromycin (2 µg/mL, #P8833, Sigma-Aldrich, St. Louis, MO, USA) for 3 days. Knockdown efficiency was confirmed using qRT-PCR and Western blot analyses. For transient Nrf2 overexpression, cells were transfected with 2 µg plasmid per well (#V79020, Invitrogen, Carlsbad, CA, USA) using Lipofectamine™ 3000 (#L3000008, Thermo Fisher Scientific, Waltham, MA, USA), followed by medium replacement with high-glucose DMEM (#C11995500BT, Gibco, Grand Island, NY, USA) after 6 h. Transfection efficiency was evaluated by qRT-PCR and Western blot analyses 48 h post-transfection.

Cell Viability Assay

U87 and U251 cell proliferation was assessed with the Cell Counting Kit-8 (CCK-8, #CK04, Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plates at 3 $\times 10^3$ cells/well in 100 µL complete DMEM and incubated overnight. Cells were then subjected to the indicated treatments for 0, 12, 24, and 48 h prior to the CCK-8 assay.

After treatment, 10 µL CCK-8 reagent was added per well, followed by 2 h incubation at 37 °C. Absorbance at 450 nm was read using a Synergy HTX microplate reader (BioTek, Winooski, VT, USA).

EdU Assay

Cell proliferation was evaluated using the 5-ethynyl-2'-deoxyuridine (EdU) assay kit (#C0071S, Beyotime, Shanghai, China). U87 and U251 cells were seeded in 24-well plates at 5×10^4 cells/well and incubated overnight. After treatments or transfections, cells were labeled with 50 µM EdU for 2 h at 37 °C. Cells were then fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 for 10 min, and EdU incorporation was visualized using the EdU detection reagent. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (#C1005, Beyotime, Shanghai, China) for 10 min. Fluorescence microscopy (Olympus IX73, Olympus, Tokyo, Japan) was used to image cells, and the percentage of EdU-positive cells was calculated from five randomly selected fields per well.

Wound Healing Assay

U87 and U251 cells were grown to near confluence in 6-well plates, scratched with a sterile pipette tip, washed with phosphate-buffered saline (PBS), and maintained in serum-free DMEM to limit proliferation effects.

Wound images were captured at 0 and 24 h using an inverted microscope (Olympus IX73), and wound width was measured with ImageJ software (NIH, Bethesda, MD, USA). The migration rate was calculated as follows:

Wound gap (%) = (wound width at indicated time point / initial wound width) × 100%.

Transwell Invasion Assay

Cell invasion was assessed using Matrigel-coated Transwell chambers with 8-µm pores (#354480, Corning, NY, USA). U87 and U251 cells (1×10^5/200 µL serum-free DMEM) were seeded in the upper chambers (50 µL Matrigel, 1:8, #356234, Corning, NY, USA), with the lower chambers containing 600 µL DMEM plus 10% FBS (#10099141C, Gibco, Grand Island, NY, USA). After 24 h at 37 °C, invading cells were fixed with 4% paraformaldehyde (#P0099, Beyotime, Shanghai, China) and stained with 0.1% crystal violet (#C0121, Beyotime, Shanghai, China). Cells in five random fields were imaged using Olympus IX73 (Tokyo, Japan) and quantified using ImageJ software (v1.53t, National Institutes of Health, Bethesda, MD, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from U87 and U251 cells was isolated using TRIzol reagent (#15596018, Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (#RR037A, Takara, Dalian, China). Quantitative PCR was performed with SYBR Green PCR Master Mix (#Q111-02, Vazyme, Nanjing, China) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 40 cycles of 95 °C for 5 s and 60 °C for 30 s after initial denaturation at 95 °C for 30 s. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method with GAPDH as reference. Primer sequences are listed in Table 1.

Western Blot Analysis

Proteins from U87 and U251 cells were extracted with RIPA buffer (#P0013B, Beyotime, Shanghai, China), quantified by BCA (#23225, Thermo Fisher Scientific), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes (#IPVH00010, Millipore, Burlington, MA, USA). After blocking, membranes were incubated with antibodies against HEATR1 (#ab241610, Abcam, Cambridge, UK), Nrf2 (#ab62352, Abcam, Cambridge, UK), and GAPDH (#ab8245, Abcam, Cambridge, UK), followed by horseradish peroxidase (HRP)-conjugated secondaries (#ab205718, #ab205719, Abcam, Cambridge, UK). Bands were visualized by enhanced chemiluminescence (#WBKLS0100, Millipore, Burlington, MA, USA) and analyzed with ImageJ (NIH, Bethesda, MD, USA). For quantitative analysis, band intensities were measured using ImageJ software (NIH, Bethesda, MD, USA), normalized to the corresponding internal control (GAPDH), and expressed as relative protein levels.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) from a minimum of three independent experiments. Statistical analyses were carried out using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). Data distribution was assessed for normality using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. Comparisons between two groups were performed using an unpaired two-tailed Student's t-test, whereas differences among three or more groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value below 0.05 was considered statistically significant.

Results

HEATR1 Is Upregulated in GBM and Efficiently Silenced by shRNA In Vitro

Using the GEPIA2 database, HEATR1 expression was analyzed across various cancers, revealing significantly higher expression in tumor tissues compared with corresponding normal tissues ($p < 0.05$, Fig. 1a). Specifically, HEATR1 was substantially overexpressed in glioblastoma samples ($p < 0.05$, Fig. 1b). Immunohistochemical im-

Table 1. Primer sequence.

Gene	Accession Number	Sequence (5′-3′)
HEATR1	NM_018072	F: GTCCGAATAGAACTGGAGCCAC R: GCCAGTAAGAACCTCCAACTTCC
Nrf2	NM_006164	F: CACATCCAGTCAGAAACCAGTGG R: GGAATGTCTGCGCCAAAAGCTG
GAPDH	NM_002046	F: GTCTCCTCTGACTTCAACAGCG R: ACCACCCTGTTGCTGTAGCCAA

HEATR1, HEAT repeat-containing protein 1; *Nrf2*, nuclear factor erythroid 2-related factor 2; *GAPDH*, Glyceraldehyde-3-Phosphate Dehydrogenase.

ages from the HPA revealed that HEATR1 staining was minimal or undetectable in brain tissue from individuals without neurological disease (Fig. 1c), while GBM specimens exhibited pronounced cytoplasmic immunoreactivity (Fig. 1d).

To validate these observations *in vitro*, HEATR1 expression was examined in normal human astrocytes (NHA) and GBM cell lines A172, U87, and U251. Western blot analysis demonstrated a pronounced upregulation of HEATR1 protein in all glioblastoma cell lines compared to NHA ($p < 0.001$, Fig. 1e,f), and qRT-PCR confirmed elevated HEATR1 mRNA levels in these cells ($p < 0.001$, Fig. 1g). Notably, U87 and U251 displayed the highest expression and were therefore selected for subsequent experiments.

To assess knockdown efficiency, U87 and U251 cells were transduced with shRNAs targeting HEATR1. HEATR1 protein levels in U87 cells were significantly diminished following shHEATR1 transduction compared with control cells, accompanied by a corresponding decrease in mRNA expression ($p < 0.01$, Fig. 1h–j). Likewise, shRNA-mediated knockdown in U251 cells led to significant reductions in both HEATR1 protein and mRNA levels ($p < 0.01$, Fig. 1k–m).

These results indicate that HEATR1 is aberrantly overexpressed in GBM and can be effectively silenced by shRNA *in vitro*.

Knockdown of HEATR1 Inhibits the Proliferation of GBM Cells

To determine the consequences of HEATR1 knockdown on the proliferative capacity of GBM cells, CCK-8 assays were conducted on U87 and U251 cells at 12, 24, and 48 hours following the knockdown treatment. Compared with the shCtrl group, U87 cells transduced with shHEATR1-1 and shHEATR1-2 exhibited significantly decreased cell viability at 48 h ($p < 0.001$, Fig. 2a). A comparable suppressive effect on cell proliferation was also observed in U251 cells ($p < 0.001$, Fig. 2b).

To further confirm these findings, EdU incorporation assays were performed. In U87 cells, the fraction of EdU-positive cells was markedly decreased in the shHEATR1 group relative to the shCtrl group ($p < 0.01$, Fig. 2c,d). Comparable reductions were detected in U251 cells, where silencing of HEATR1 led to a significant reduction in EdU incorporation ($p < 0.01$, Fig. 2e,f).

These results suggest that silencing HEATR1 significantly inhibits the proliferative potential of GBM cells *in vitro*.

Silencing HEATR1 Inhibits the Migratory and Invasive Abilities of GBM Cells

To explore how silencing HEATR1 influences the migratory behavior of GBM cells, wound healing assays were performed in U87 and U251 lines, with images captured at baseline and 24 hours following scratch creation. Compared with the shCtrl group, U87 cells transduced with shHEATR1 exhibited a significantly higher remaining wound area at 24 hours, indicating impaired migratory ability ($p < 0.01$, Fig. 3a,b). Similarly, HEATR1 silencing in U251 cells increased the proportion of unhealed wound area at 24 hours ($p < 0.01$, Fig. 3c,d).

Furthermore, Transwell invasion assays demonstrated that the invasive ability of U87 cells was substantially decreased following HEATR1 silencing compared to the control group ($p < 0.001$, Fig. 3e,f). Parallel results were observed in U251 cells, in which the invasive ability was markedly reduced following HEATR1 silencing ($p < 0.01$, Fig. 3g,h).

Overall, the experimental evidence indicates that knockdown of HEATR1 significantly compromises both the migratory and invasive potential of GBM cells in culture.

HEATR1 Knockdown Is Associated With Reduced Nrf2 Expression in GBM

Based on preliminary experiments, shHEATR1-1 was selected for subsequent studies due to its superior knockdown efficiency and its more pronounced inhibitory effect on the malignant progression of GBM cells.

To explore the potential relationship between HEATR1 and Nrf2, correlation analysis was performed using data obtained from the cBioPortal database. Analysis indicated a positive association between the expression of HEATR1 and Nrf2 in GBM (Fig. 4a). Furthermore, evaluation using GEPIA demonstrated that Nrf2 was sig-

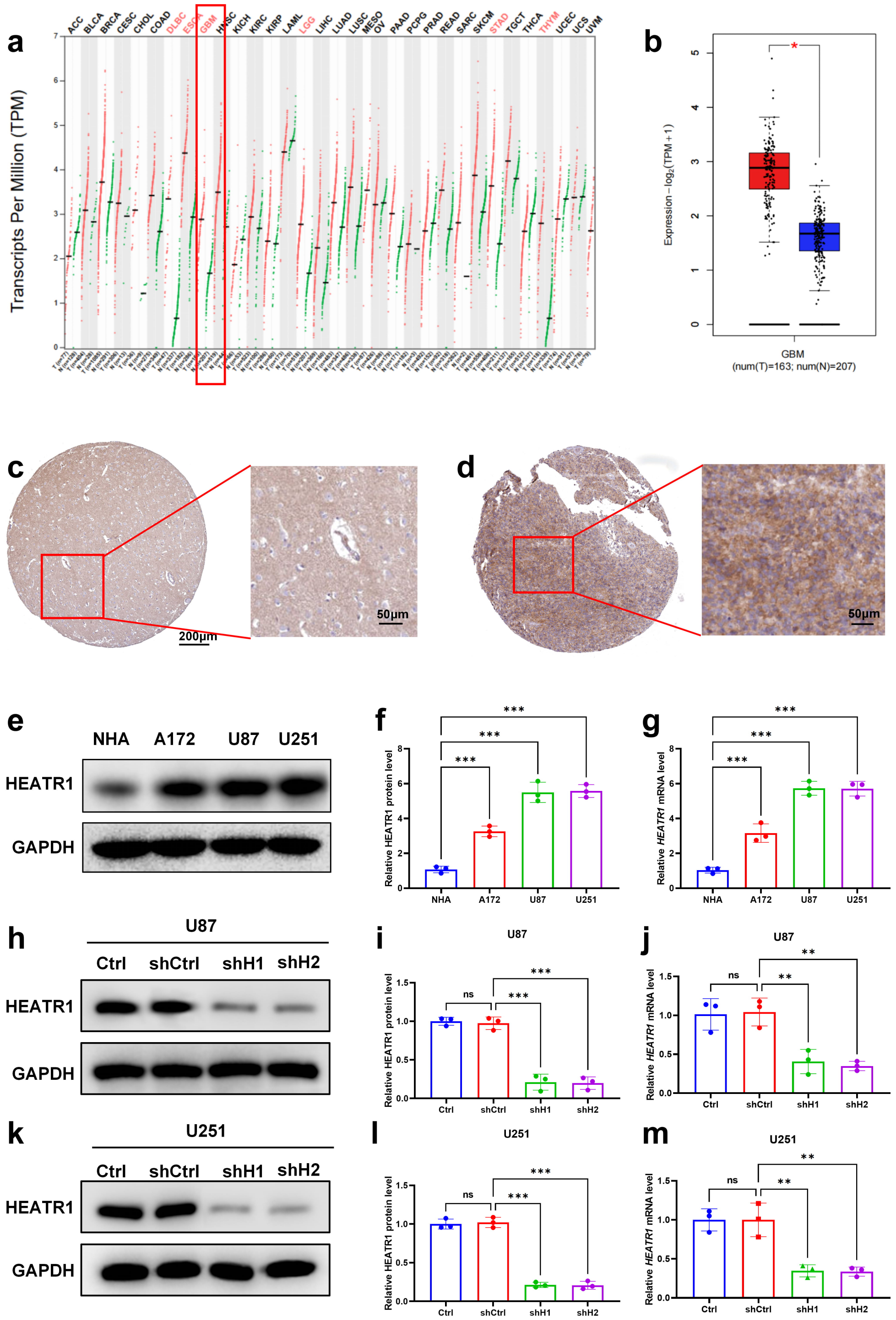

Fig. 1. HEATR1 expression and shRNA knockdown in GBM. (a) HEATR1 expression levels in various tumor types and normal tissues, analyzed using the GEPIA2 online tool. (b) Comparison of HEATR1 expression in GBM and normal brain tissues (GEPIA2), T: tumor, N: normal. (c,d) Representative immunohistochemical staining of HEATR1 in normal brain tissue (c) and GBM tissue (d) from the HPA database. (e,f) Western blot analysis of HEATR1 protein expression in NHA, A172, U87, and U251 cell lines. (g) Quantitative real-time PCR analysis of HEATR1 mRNA levels in NHA and GBM cell lines. U87 and U251 cells were transduced with either shCtrl or shHEATR1 and shHEATR1-2. (h,i) Western blot analysis of HEATR1 expression in U87 cells. (j) qRT-PCR analysis of *HEATR1* mRNA levels in U87 cells. (k,l) Western blot analysis of HEATR1 expression in U251 cells. (m) qRT-PCR analysis of *HEATR1* mRNA levels in U251 cells. Data are presented as mean ± SD (n = 3). ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. HEATR1, HEAT repeat-containing protein 1; GBM, Glioblastoma multiforme; GEPIA2, Gene Expression Profiling Interactive Analysis 2; HPA, Human Protein Atlas; NHA, Normal human astrocytes; SD, standard deviation.

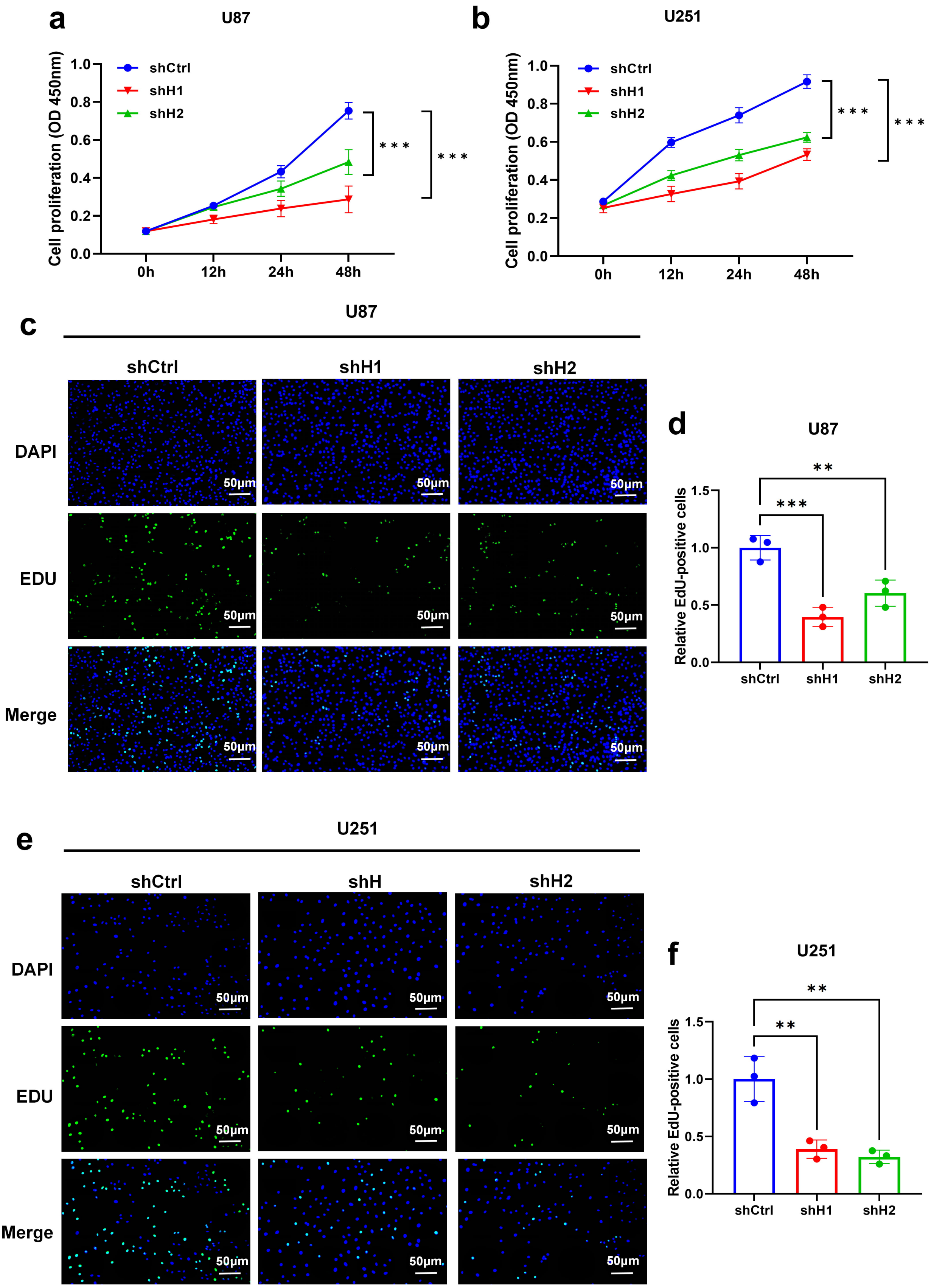

Fig. 2. HEATR1 knockdown inhibits the proliferation of GBM cells. (a) CCK-8 assay of U87 cells at 12, 24, and 48 hours. (b) CCK-8 assay of U251 cells at 12, 24, and 48 hours. (c,d) EdU staining of U87 cells. (e,f) EdU staining of U251 cells. Data are presented as mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$. CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine.

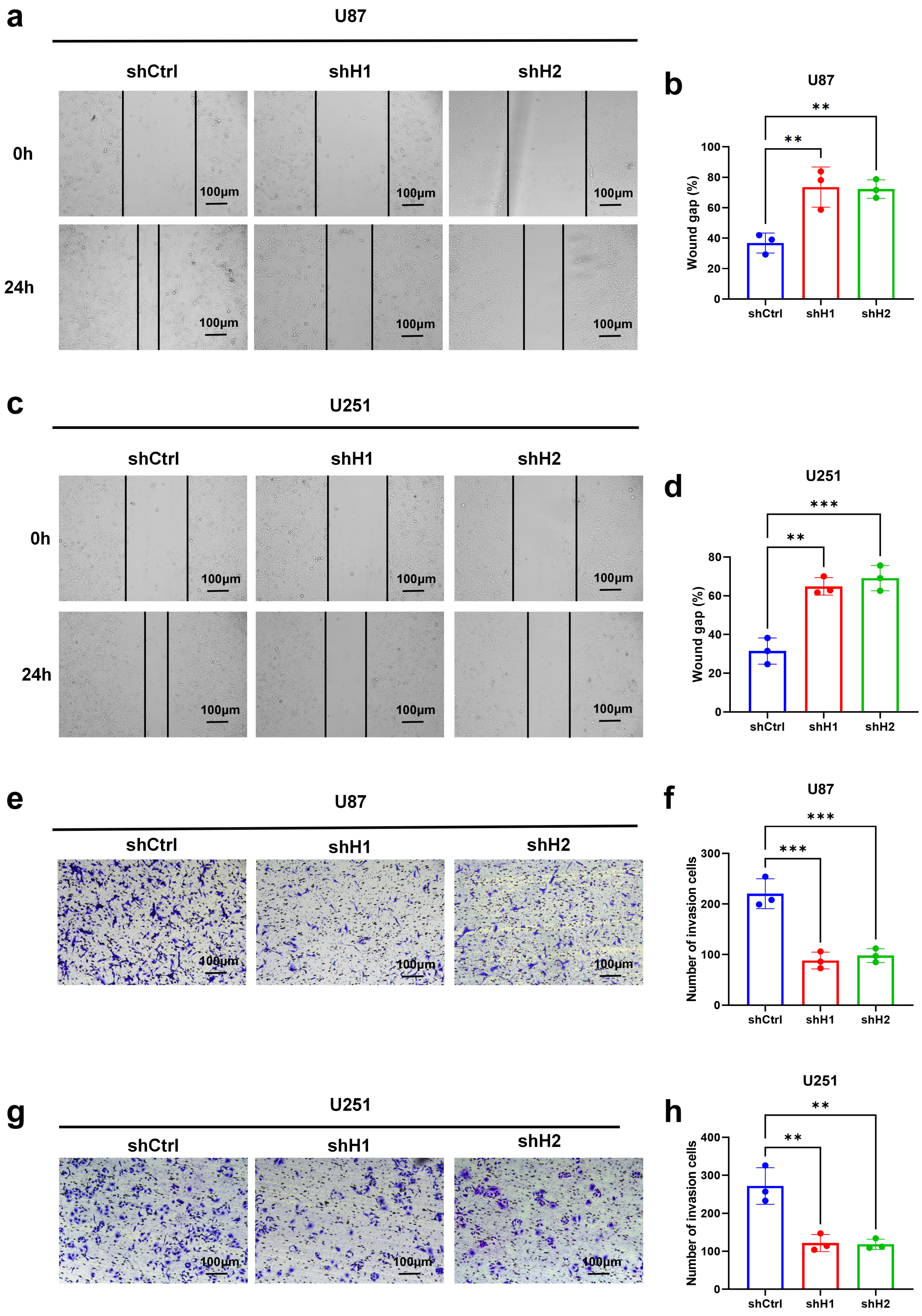

Fig. 3. HEATR1 knockdown impairs the migration and invasion of GBM cells. U87 and U251 cells were transduced with shCtrl or shHEATR1-1 and shHEATR1-2. (a,b) Wound healing assay of U87 cells at 0 and 24 hours. (c,d) Wound healing assay of U251 cells at 0 and 24 hours. (e,f) Transwell invasion assay of U87 cells. (g,h) Transwell invasion assay of U251 cells. Data are presented as mean ± SD (n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$.

nificantly overexpressed in glioblastoma tissues compared with normal brain regions ($p < 0.05$, Fig. 4b).

To examine the potential modulatory effect of HEATR1 on Nrf2, Nrf2 expression levels were assessed in GBM cells after silencing HEATR1. Western blotting revealed that Nrf2 expression in U87 cells was substantially diminished after HEATR1 knockdown ($p < 0.001$, Fig. 4c,d) and U251 cells ($p < 0.01$, Fig. 4f,g). Similarly, qRT-PCR analysis confirmed that Nrf2 mRNA expression was substantially decreased in U87 ($p < 0.05$, Fig. 4e) and U251 cells ($p < 0.001$, Fig. 4h) upon HEATR1 knockdown.

These data suggest that HEATR1 knockdown is associated with decreased Nrf2 expression at both the mRNA and protein levels in GBM cells.

HEATR1 Modulates Nrf2 Expression but Is Not Affected by Nrf2

To verify the efficiency of Nrf2 modulation, U87 and U251 cells were transduced with Nrf2-targeting shRNA (shN1 and shN2). Western blot and qRT-PCR analyses revealed that Nrf2 protein and mRNA were significantly diminished in shN1 and shN2 cells ($p < 0.01$, Fig. 5a–f). Among them, shN2 exhibited a stronger knockdown efficiency and was therefore selected for subsequent experiments.

Nrf2 overexpression efficiency was validated by both qRT-PCR and Western blot analyses, which demonstrated that *Nrf2* mRNA and Nrf2 protein levels were significantly elevated in oeN cells compared with control and shCtrl groups ($p < 0.001$, Fig. 5g–i), confirming the successful establishment of the Nrf2 overexpression model.

To elucidate the interaction between HEATR1 and Nrf2, the levels of both proteins were analyzed in U87 and U251 cells under distinct experimental conditions, including HEATR1 knockdown (shH), Nrf2 knockdown (shN), Nrf2 overexpression (oeN), and combined treatments (shH + shN, shH + oeN). This experimental setup enabled the assessment of both the individual and combined effects of HEATR1 and Nrf2 modulation.

Western blot analysis revealed that Nrf2 knockdown or overexpression alone did not significantly affect HEATR1 protein levels ($p > 0.05$), indicating that Nrf2 is not a regulator of HEATR1. In contrast, knockdown of HEATR1 resulted in a significant reduction in Nrf2 expression in both U87 and U251 cell lines ($p < 0.05$). Notably, simultaneous overexpression of Nrf2 in cells with HEATR1 knockdown (shH + oeN) partially restored Nrf2 protein levels compared to cells treated with shH alone. indicating that Nrf2 overexpression partially rescued the suppression induced by HEATR1 knockdown ($p < 0.001$, Fig. 5j–s).

In summary, the findings reveal that HEATR1 upregulates Nrf2 expression in GBM cells, while Nrf2 does not appear to exert any feedback regulation on HEATR1.

Nrf2 Reverses HEATR1 Knockdown–Mediated Proliferation Suppression

To assess whether overexpression of Nrf2 can mitigate the inhibitory impact of HEATR1 knockdown on the proliferative capacity of GBM cells, U87 and U251 cells were treated with shCtrl, HEATR1 knockdown (shH), combined HEATR1 and Nrf2 knockdown (shH + shN), or HEATR1 knockdown plus Nrf2 overexpression (shH + oeN).

CCK-8 assays demonstrated that silencing HEATR1 markedly decreased cell viability at 48 hours in both U87 ($p < 0.05$, Fig. 6a) and U251 cells ($p < 0.001$, Fig. 6b) relative to the shCtrl group. Combined knockdown of HEATR1 and Nrf2 (shH + shN) further decreased proliferation compared to shH alone ($p < 0.05$). In contrast, Nrf2 overexpression partially reversed the proliferation inhibition induced by HEATR1 knockdown (shH + oeN) in both cell lines ($p < 0.01$).

EdU incorporation assays confirmed these findings, showing a decreased proportion of EdU-positive cells in the shH groups in U87 ($p < 0.001$, Fig. 6c,d) and U251 cells ($p < 0.001$, Fig. 6e,f). Nrf2 overexpression partially restored DNA synthesis in HEATR1-silenced cells ($p < 0.05$).

These findings indicate that Nrf2 can partially counteract the inhibitory effect of HEATR1 knockdown on GBM cell proliferation.

Nrf2 Reverses HEATR1 Knockdown–Mediated Migration and Invasion Suppression

To determine whether forced Nrf2 expression can counteract the suppressive effects of HEATR1 knockdown on GBM cell migration and invasion, U87 and U251 cells received treatment with shCtrl, HEATR1 knockdown (shH), combined HEATR1 and Nrf2 knockdown (shH + shN), or HEATR1 knockdown plus Nrf2 overexpression (shH + oeN).

Wound healing assays showed that HEATR1 depletion considerably reduced U87 cell migration at 24 hours compared to cells treated with shCtrl ($p < 0.01$, Fig. 7a,b). A similar inhibitory effect was observed in U251 cells under the same conditions ($p < 0.01$, Fig. 7c,d). Combined knockdown of HEATR1 and Nrf2 (shH + shN) further inhibited migration compared to shH alone ($p < 0.01$). Conversely, Nrf2 overexpression partially reversed the migration defect induced by HEATR1 knockdown (shH + oeN) in both cell lines ($p < 0.05$).

Transwell invasion experiments further validated these findings, demonstrating that HEATR1 knockdown significantly decreased the invasive capacity of U87 cells ($p < 0.001$, Fig. 7e,f). A comparable reduction in invasion was observed in U251 cells ($p < 0.001$, Fig. 7g,h). Combined knockdown of HEATR1 and Nrf2 further suppressed invasion ($p < 0.05$), while Nrf2 overexpression partially restored the invasive capacity in HEATR1 knockdown cells ($p < 0.05$).

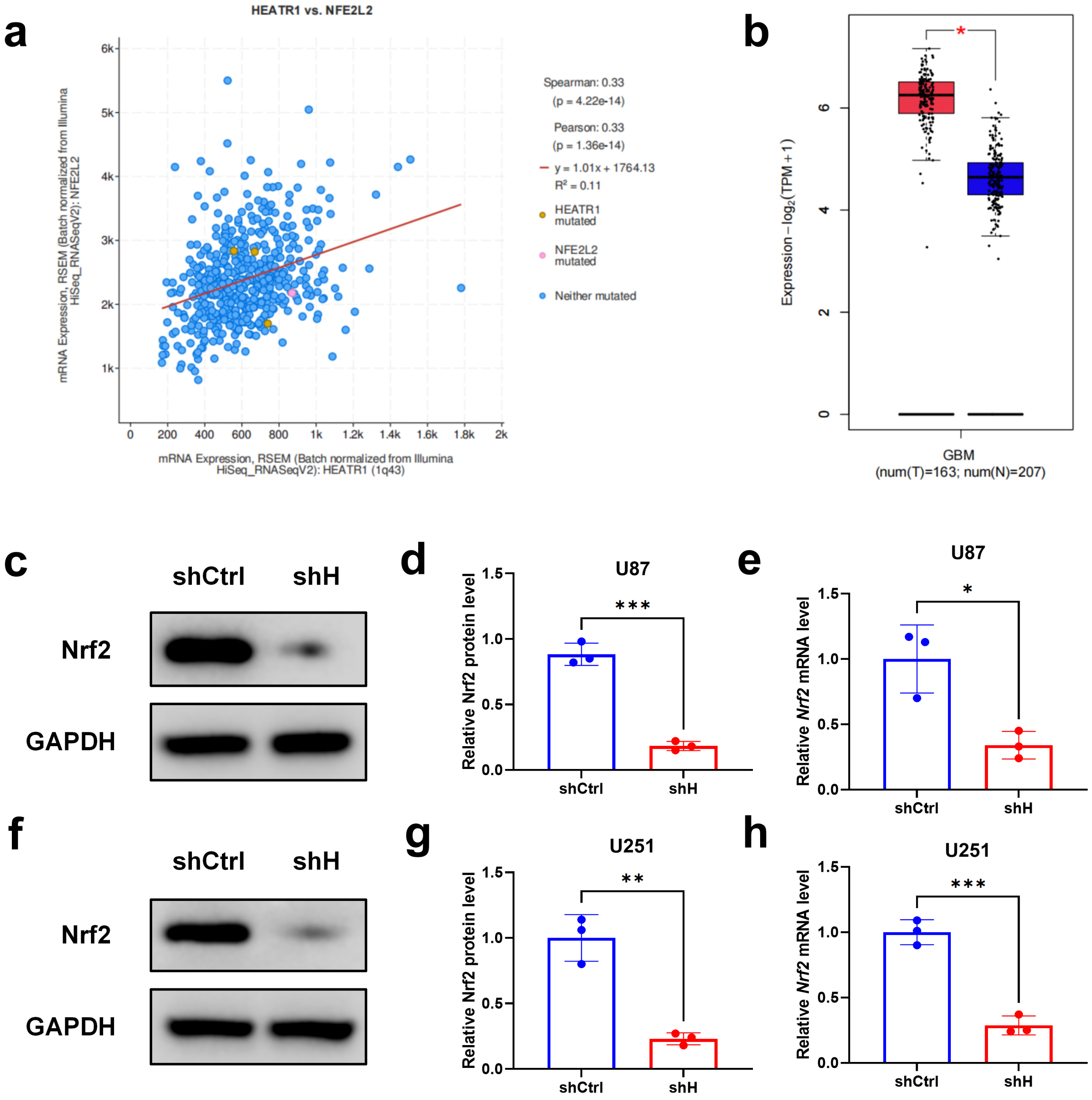

Fig. 4. HEATR1 knockdown reduces Nrf2 expression in GBM cells. (a) Correlation analysis between HEATR1 and Nrf2 expression in GBM (cBioPortal). (b) Nrf2 expression in GBM and normal brain tissues (GEPIA), T: tumor, N: normal. (c,d) Western blot analysis of Nrf2 protein levels in U87 cells. (e) qRT-PCR analysis of *Nrf2* mRNA levels in U87 cells. (f,g) Western blot analysis of Nrf2 protein levels in U251 cells. (h) qRT-PCR analysis of *Nrf2* mRNA levels in U251 cells. Data are presented as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Nrf2, nuclear factor erythroid 2-related factor 2.

These findings indicate that Nrf2 partially rescues the suppressive effects of HEATR1 knockdown on GBM cell migration and invasion.

Discussion

GBM is the most aggressive subtype of primary brain tumors, and patients diagnosed with this tumor type usually exhibit extremely poor clinical outcomes and shortened survival expectations. The pronounced invasiveness of GBM, combined with its marked resistance to standard treatments, underscores the urgent need to identify new molecular drivers and potential therapeutic targets. HEATR1, a protein characterized by HEAT repeat domains involved in intracellular transport and signal transduction, has been documented in the literature to exert oncogenic capacities across multiple cancers, contributing to both tumor

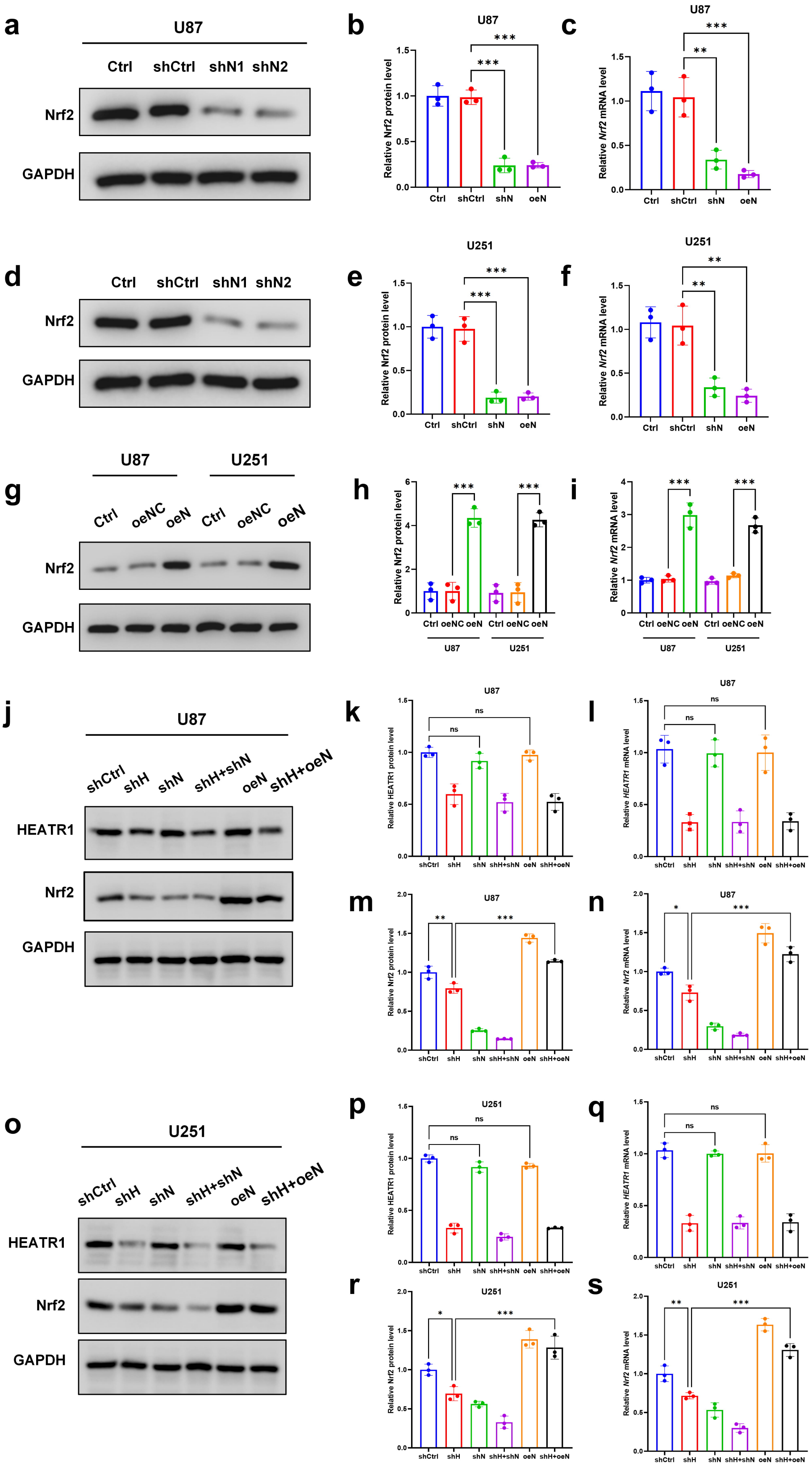

Fig. 5. Nrf2 modulation efficiency and interaction with HEATR1 in GBM cells. (a,b) Nrf2 protein levels in U87 cells with Ctrl, shCtrl, shN1, or shN2. (c) Nrf2 mRNA levels in U87 cells. (d,e) Nrf2 protein levels in U251 cells with the same treatments. (f) Nrf2 mRNA levels in U251 cells. (g–i) U87 and U251 cells were transduced with Ctrl or oeNC and oeN. (j–n) HEATR1 and Nrf2 protein levels in U87 cells with shCtrl, shH, shN, shH + shN, oeN, or shH + oeN. (o–s) HEATR1 and Nrf2 protein levels in U251 cells with the same treatments. Data are presented as mean ± SD (n = 3). ns: not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

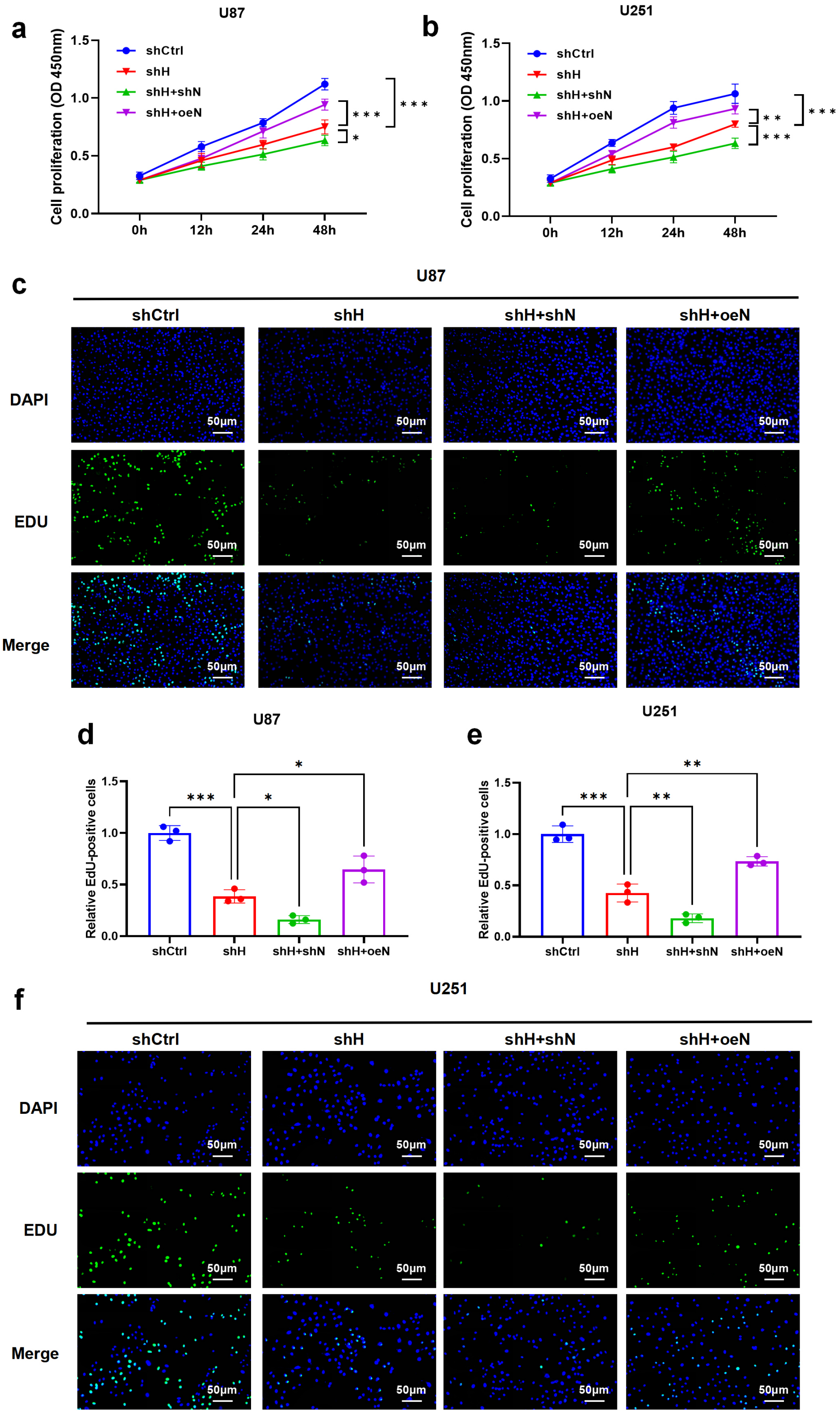

Fig. 6. Nrf2 reverses HEATR1 knockdown–mediated proliferation suppression. U87 and U251 cells were transduced with shCtrl, shH, shH + shN, or shH + oeN. (a) CCK-8 assay of U87 cells at 12, 24, and 48 hours. (b) CCK-8 assay of U251 cells at 12, 24, and 48 hours. (c,d) EdU staining of U87 cells. (e,f) EdU staining of U251 cells. Data are presented as mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

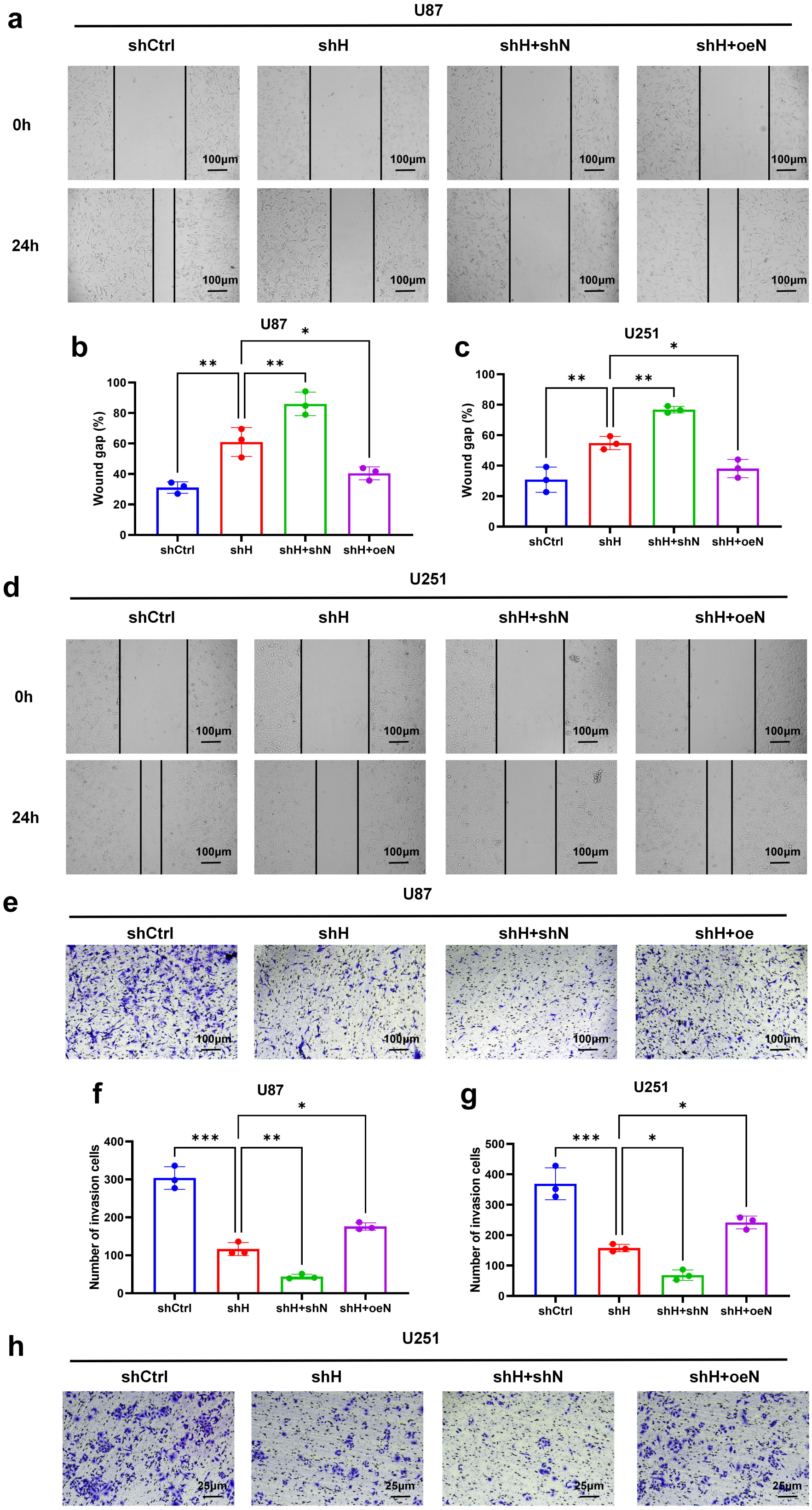

Fig. 7. Nrf2 reverses HEATR1 knockdown–mediated migration and invasion suppression. U87 and U251 cells were transduced with shCtrl, shH, shH + shN, or shH + oeN. (a,b) Scratch wound healing assay of U87 cells at 0 and 24 hours. (c,d) Scratch wound healing assay of U251 cells at 0 and 24 hours. (e,f) Transwell invasion assay of U87 cells. (g,h) Transwell invasion assay of U251 cells. Data are presented as mean ± SD (n = 3). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

development and metastatic progression [4,11,12]. Given these findings, exploring the role of HEATR1 in GBM is of significant value to understand its contribution to tumor progression and identify potential therapeutic targets. Our investigation assessed HEATR1 expression and its biological implications in glioblastoma, uncovering its pivotal role in orchestrating malignant cell proliferation, migration, and invasion.

Our bioinformatics analysis from GEPIA2 and cBioPortal, combined with experimental validation, confirms elevated HEATR1 expression in GBM, consistent with findings in other malignancies [4]. Previous studies have linked HEATR1 to cancer progression by regulating cellular stress responses and protein homeostasis, yet its molecular mechanisms remain largely unclear. The observed suppression of GBM cell proliferation and DNA synthesis upon HEATR1 knockdown aligns with these reports and underscores its critical role in maintaining aggressive tumor phenotypes [13].

A major and novel finding from our work is that HEATR1 positively regulates Nrf2, a pivotal transcription factor that orchestrates cellular antioxidant responses and ensures the maintenance of redox balance within cells. Nrf2 exerts a dual function in cancer biology. Under physiological homeostasis, the transcription factor Nrf2 acts as a critical cytoprotective mediator, mitigating oxidative insults and cellular stress through the upregulation of diverse genes responsible for antioxidant defense and detoxifying processes. In contrast, in various malignancies, including glioblastoma, persistent or dysregulated activation of Nrf2 has been reported to facilitate tumor-promoting processes, such as increased cell survival, accelerated proliferation, enhanced metastatic potential, and elevated resistance to chemotherapeutic and radiotherapeutic interventions [14–16]. For example, recent research has shown that increased Nrf2 activity supports the maintenance of GBM stem-like cell populations and enhances resistance to therapies, thereby contributing to tumor aggressiveness and unfavorable clinical outcomes [17,18].

In this study, depletion of HEATR1 in GBM cells resulted in significantly diminished Nrf2 expression, reflected by reduced mRNA and protein levels, suggesting a regulatory role of HEATR1 in sustaining Nrf2 activity. This suggests that HEATR1 may influence Nrf2 either at the transcriptional level or through post-transcriptional mechanisms affecting mRNA stability or protein turnover. Interestingly, altering Nrf2 expression alone—through either knockdown or overexpression—did not affect HEATR1 levels, indicating a unidirectional regulatory axis in which HEATR1 acts upstream of Nrf2. Such a relationship reveals a previously unrecognized layer of Nrf2 regulation in GBM.

Furthermore, functional rescue assays demonstrated that Nrf2 overexpression partially restored the proliferation, migration, and invasion of glioblastoma cells suppressed by HEATR1 knockdown. This finding firmly positions Nrf2 as a critical downstream effector mediating the oncogenic functions of HEATR1 in GBM cells. It also highlights the HEATR1-Nrf2 axis as a potential vulnerability in GBM that could be therapeutically targeted.

Mechanistically, the HEATR1-Nrf2 axis likely facilitates GBM progression by enhancing cellular antioxidant capacity, protecting cells from oxidative stress-induced apoptosis, and promoting invasive behaviors. This is supported by previous studies linking Nrf2 activation to increased matrix metalloproteinase expression and epithelial-mesenchymal transition in GBM [19,20]. Nevertheless, the precise molecular interactions between HEATR1 and Nrf2 remain to be defined. HEATR1 may modulate upstream signaling pathways, transcriptional coactivators, or RNA-binding proteins that control Nrf2 expression. Techniques such as chromatin immunoprecipitation sequencing (ChIP-seq), RNA immunoprecipitation, and proteomic profiling will be instrumental to elucidate this regulatory mechanism.

Overall, the present research uncovers a novel tumor-promoting role of HEATR1 in glioblastoma, showing that it enhances cell proliferation, migration, and invasion via the upregulation of Nrf2. Overall, these observations advance our understanding of GBM and position the HEATR1-Nrf2 pathway as a candidate for future therapeutic development. Future work should validate these findings *in vivo*, explore clinical correlations with patient prognosis, and dissect the detailed molecular interplay between HEATR1 and Nrf2, with the aim of facilitating translation into clinical utility.

Conclusion

In summary, this study identifies HEATR1 as an oncogenic factor in the proliferation, migration, and invasion of GBM cells. Mechanistically, HEATR1 functions upstream of Nrf2, sustaining its expression and activity, and thereby enhancing cellular antioxidant defenses and malignant behaviors. Functional rescue experiments confirm that Nrf2 mediates the tumor-promoting effects of HEATR1, highlighting a unidirectional HEATR1–Nrf2 regulatory axis. These findings advance our understanding of GBM pathobiology and suggest that targeting the HEATR1–Nrf2 pathway may represent a promising therapeutic strategy. Future studies should validate this axis in vivo, exploring its clinical relevance and elucidating the molecular mechanisms linking HEATR1 to Nrf2 regulation.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

XX and HZ designed the study and contributed to conceptualization and methodology. ND conducted data collection, investigation, validation, and visualization. DW performed formal analysis and provided software support. CT contributed to the conception and design of the study, participated in data interpretation, provided resources, supervised the study, and contributed to project administration. XX drafted the original manuscript. All authors contributed to critical revision of the manuscript for important intellectual content. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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