



The Tumor Suppressor Role of Zinc Finger Protein 671 (ZNF671) in Multiple Tumors Based on Cancer Single-Cell Sequencing

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In humans, zinc finger protein 671 (ZNF671) is a type of transcription factor. However, the contribution of tumor heterogeneity to the functional role of ZNF671 remains unknown. The present study aimed to determine the functional states of ZNF671 in cancer single cells based on single-cell sequencing datasets (scRNA-seq). We collected cancer-related ZNF671 scRNA-seq datasets and analyzed ZNF671 in the datasets. We evaluated 14 functional states of ZNF671 in cancers and performed ZNF671 expression and function state correlation analysis. We further applied t-distributed stochastic neighbor embedding to describe the distribution of cancer cells and to explore the functional state of ZNF671 in cancer subgroups. We found that ZNF671 was downregulated in eight cancer-related ZNF671 scRNA-seq datasets. Functional analysis identified that ZNF671 might play a tumor suppressor role in cancer. The heterogeneous functional states of cell subgroups and correlation analysis showed that ZNF671 played tumor suppressor roles in heterogeneous cancer cell populations. Western blot and transwell assays identified that ZNF671 inhibited EMT, migration, and invasion of CNS cancers, lung cancer, melanoma, and breast carcinoma *in vitro*. These results from cancer single-cell sequencing indicated that ZNF671 played a tumor suppressor role in multiple tumors and may provide us with new insights into the role of ZNF671 for cancer treatment.

Keywords: ZNF671, tumor suppressor, solid tumor, single-cell sequencing, data mining

INTRODUCTION

Cancer is a complex ecosystem composed of cells with heterogeneous functional states, leading to both therapeutic resistance, and frequent cancer recurrence or metastasis, which poses a major obstacle to cancer diagnosis and treatment (1–3). Some tumor cells have high proliferative or apoptotic capacity, some have invasion and metastasis activities, some show stem-like properties, and some exhibit a quiescent state (4, 5). These functionally heterogeneous cancer cells act cooperatively or competitively during tumor progression or metastasis, leading to distinct tumor phenotypes (6–8). Therefore, it is essential to systematically and comprehensively identify the functional states of cancer cells.

Single-cell mRNA-sequencing (scRNA-seq) provides a powerful tool for characterizing the omic-scale features of heterogeneous cell populations (9, 10). ScRNA-seq technologies permit the dissection of primary tumor cells, metastatic tumor cells, cancer stem cells (CSC), circulating tumor cells (CTC), and disseminated tumor cells in a comprehensive and unbiased manner, with no need of any prior knowledge of the cell population. ScRNA-seq has become a reference tool for analyzing the composition of cancer tissues and for establishing the characteristics of the cellular microenvironment (11). Thus, understanding single cancer cells will advance our understanding of not only therapeutic resistance but all facets of cell biology. Furthermore, the application of scRNA-seq in the clinic has the potential to change our approach to cancer management fundamentally (12).

Zinc finger protein 671 (*ZNF671*) is a member of the KRAB-ZFP (KRAB-ZFP) family of mammalian transcriptional repressors (13–15). KRAB-ZFPs can regulate tumor cell differentiation, proliferation, apoptosis, invasion, metastasis, and transformation (16–21). Previous studies showed that *ZNF671* could act as a tumor suppressor in several solid tumors (22–26). Our studies identified that *ZNF671* played a tumor suppressor role in breast invasive carcinoma (BRCA), cervical squamous cell carcinoma, and endocervical adenocarcinoma (CESC), head and neck squamous cell carcinoma (HNSC), kidney renal papillary cell carcinoma (KIRP), lung adenocarcinoma (LUAD), pancreatic adenocarcinoma (PAAD), and uterine corpus endometrial carcinoma (UCEC) (26, 27). However, the roles of *ZNF671* in the functional heterogeneity of cancer single cells remain unclear.

In this study, we analyzed the expression of *ZNF671* in cancer scRNA-seq datasets systematically. We explored the functional role of *ZNF671* in solid tumors and analyzed its expression and functional correlation in tumors. We further described the distribution of cancer single cells and explored their functional relevance in different tumor cell subgroups. Our results provide important insights into tumor heterogeneity and enhance knowledge of the tumor suppressor role of *ZNF671* in solid tumors.

MATERIALS AND METHODS

Data Collection

Data were collected based on the following keywords: (“single cells” OR “single cell” OR “single-cell” OR “single-cells”) AND (“transcriptome” OR “transcriptomics” OR “scRNA-seq” OR “scRNA seq” OR “RNA-sequencing” OR “RNA-seq” OR “RNA sequencing”) AND (“carcinoma” OR “tumor” OR “tumor” OR “cancer” OR “neoplasm” OR “neoplastic”). According to the method used by Yuan et al. (28), three human data sets from Array Express, Sequence Read Archive (SRA), and Gene Expression Omnibus (GEO) datasets were collected and all single-cell data in these datasets were analyzed via expression quantification, quality control, and characterization of functional states.

Data Processing

Transcript expression quantification was performed using Salmon (version 0.9.1) with the optional parameter k ($k = 31$ for long reads and $k = 15$ for short reads). The GENCODE (Release 28, GRCh38) reference transcriptome was used to detect gcBias, seqBias, and other default parameters in the quasi-mapping-based mode. For scRNA-seq datasets with only an expression matrix, we directly converted the expression values to transcripts per million (TPM)/counts per million (CPM) values using a custom script. Expression values were log₂ transformed with an offset of 1.

Characterizing Functional States of Cancer Single Cells

After reviewing cancer single-cell sequencing studies, 14 crucial functional states of cancer cells were selected, including angiogenesis, apoptosis, cell cycle, differentiation, DNA damage, DNA repair, epithelial-mesenchyme transition (EMT), hypoxia, inflammation, invasion, metastasis, proliferation, quiescence, and stemness using Gene Ontology, MSigDB, Cyclebase, HCMDB, and StemMapper (29–33). According to the method used by Yuan et al. (28), the activities of the 14 functional states across cancer single cells in the datasets were assessed using the Gene Set Variation Analysis (GSVA) package downloaded from <http://www.bioconductor.org> (34).

Dimensionality Reduction Using t-distributed Stochastic Neighbor Embedding (t-SNE) Analysis

According to the method used by Li et al. (35), donor files were imported into R, and expression matrices containing measured intensities at the single-cell level were extracted from the flowCore package. A subset of cells was selected for each donor at random and merged into a single expression matrix before t-SNE analysis. The beads, viability, center, offset, residual, event length, intercalator, and time channels were removed from the expression matrix. The *ZNF671* protein marker was the only factor included in the t-SNE analysis, and *ZNF671* intensities were transformed using the inverse hyperbolic sine (arcsinh) function.

T-SNE calculations were performed with 1,000 iterations, a perplexity parameter of 30, and a trade-off θ of 0.5, which was used to visualize similarities and the proximity of cells in a two-dimensional plot. T-SNE maps were generated by plotting each event of the t-SNE dimensions in a dot-plot. *ZNF671* intensities were overlaid on the dot-plot to show the expression in different cell islands and to facilitate the assignment of cell subsets to these islands. The t-SNE dimensions were characterized by t-SNE1 and t-SNE2 in the given graphs. The software is available at <https://github.com/KlugerLab/Flt-SNE>.

ZNF671 Expression and Functional State Correlation Analysis

The expression level statistics of *ZNF671* in each cell were converted to normalized ranks and Next, the Kolmogorov–Smirnov liker random walk statistic, similar to the GSEA

method, was used to summarize the *ZNF671* expression-level rank statistics of a given signature gene set into a final enrichment score, which was used to characterize the signature activity. The enrichments of 14 signatures across cells in the scRNA-seq data were calculated, and only cells with detectable expression of *ZNF671* were used. Correlations between *ZNF671* expression and functional state activities were assessed using correlation analysis with false discovery rate (FDR) corrections for multiple comparisons (FDR < 0.05 and $P < 0.05$).

Cell Culture

Human GBM cell lines (U87 and U251), the A375 melanoma cell line, and triple-negative breast cancer cell lines (MDA-MB-231 and BT-549) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37°C, 5% CO₂ in 10% DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Western Blot Analysis

After cells were transfected with the pEnter-*ZNF671* or pEnter-vector plasmids (Vigene Biosciences, Shandong, China) for 48 h, RIPA lysis buffer (Beyotime, Shanghai, China) was used to isolate proteins. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and incubated with primary anti-*ZNF671* (1:500; Proteintech, Chicago, IL, USA), E-cadherin (1:500, BD Biosciences), Vimtenin (1:500, BD Biosciences), and anti-GAPDH (1:1,000, Proteintech, Chicago, IL, USA).

Migration and Invasion Assays

Transwell plates (8-μm pores) (Costar/Corning, Lowell, MA) were used for Transwell migration or invasion assays. 5×10^4 (migration assay) or 1×10^5 (invasion assay) cells resuspended in serum-free medium were placed in the upper chamber of each insert, either uncoated or coated with Matrigel (BD Biosciences). The lower chamber contained culture medium with 10% FBS to act as a chemoattractant. The cells were incubated for 12 or 24 h and were then fixed and stained. Cells on the undersides of the filters were observed and counted under 200× magnification.

Statistical Analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed using the two-tailed unpaired Student's *t*-test; $P < 0.05$ was considered statistically significant.

RESULTS

scRNA-seq Dataset Features

As shown in **Table 1**, a total of eight cancer-related *ZNF671* scRNA-seq datasets were included in the study. They contained 14 functional states of 13941 cancer single cells from glioblastoma (GBM; $n = 623$), glioma (brain; $n = 2259$), glioma (PDX; $n = 167$), astrocytoma (AST; $n = 5097$), oligodendrogloma (ODG;

TABLE 1 | Features of the scRNA-seq datasets searched with *ZNF671*.

ExpID	Name	Cancer	No. Cells
0058	Patel AP. Science. 2014 (Brain)	Glioblastoma	623
0059	Filbin MG.Science. 2018 (Brain)	Glioma	2259
0060	Filbin MG.Science. 2018 (PDX)	Glioma	167
0056	Venteicher AS. Science. 2017 (Brain)	Astrocytoma	5097
0062	Tirosh I. Nature. 2016 (Brain)	Oligodendrogloma	4043
0066	Kim KT. Genome Biol. 2015 (PDX)	Lung adenocarcinoma	126
0071	Tirosh I. Science. 2016 (Skin)	Melanoma	1257
0052	Braune EB. Stem Cell Reports. 2016 (PDX)	Breast cancer	369

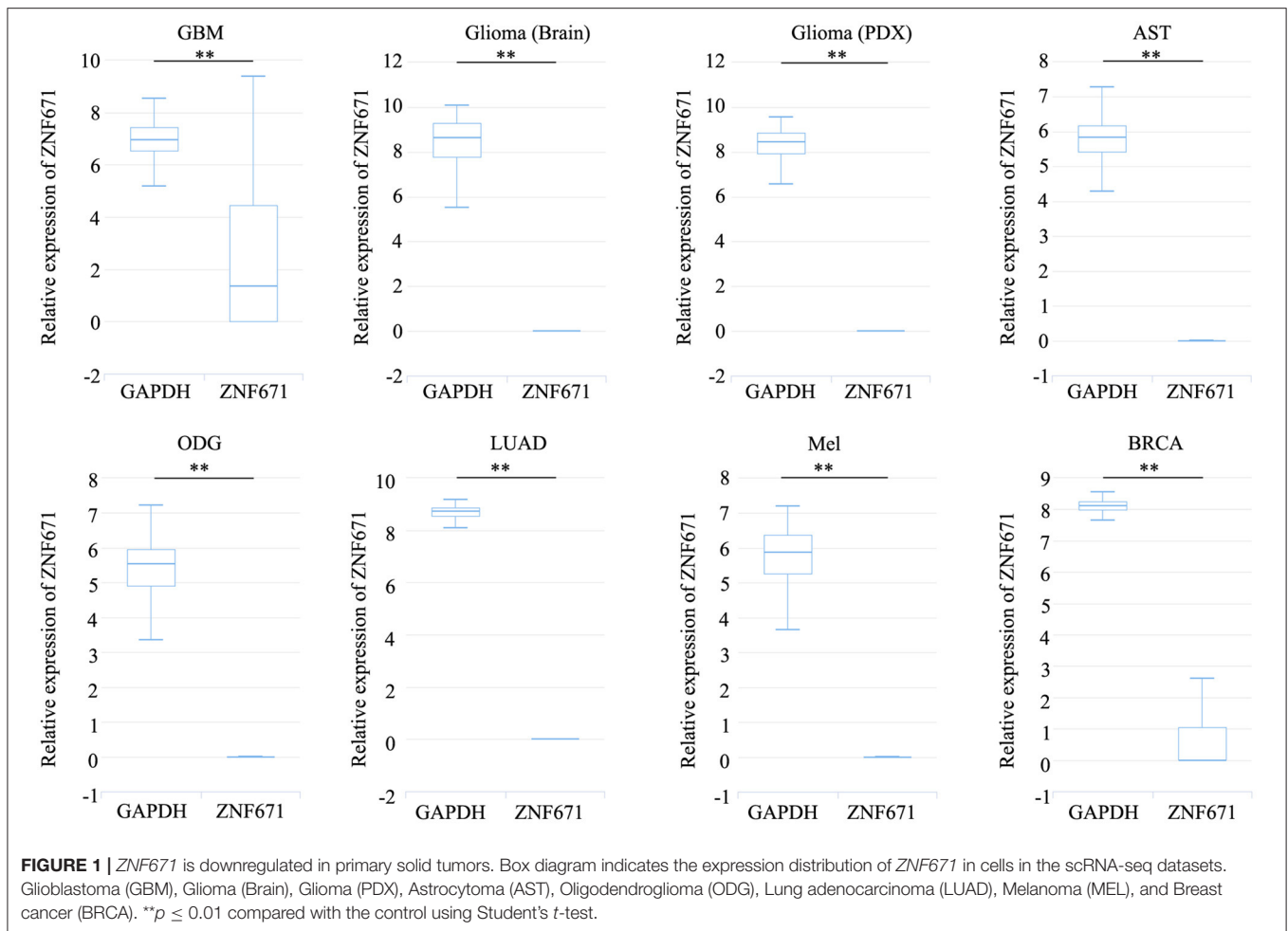
$n = 4043$), lung adenocarcinoma (LUAD; $n = 126$), melanoma (MEL; $n = 1257$), and breast cancer (BRCA; $n = 369$).

ZNF671 Functional States in the scRNA-seq Datasets

Expression analysis showed that *ZNF671* was obviously downregulated in GBM, glioma, AST, ODG, LUAD, MEL, and BRCA (**Figure 1**), which indicated that *ZNF671* might play an important role in tumor progression. To further explore the functional role of *ZNF671* in different cancers, 14 crucial functional states of cancer cells, including angiogenesis, apoptosis, cell cycle, differentiation, DNA damage, DNA repair, EMT, hypoxia, inflammation, invasion, metastasis, proliferation, quiescence, and stemness were summarized and analyzed. As shown in **Figure 2**, the expression of *ZNF671* and the activity of each functional state across single-cell datasets in different cancers were explored using an interactive bubble chart. The upper bar plot shows a summary of the association between the functional state and the number of single-cell datasets. We found that the expression of *ZNF671* had a significant negative regulation for angiogenesis, apoptosis, EMT, hypoxia, invasion, and quiescence, which was consistent with our previous research (26, 27). These results indicate that *ZNF671* might play a suppressor role in tumor development.

The Different Roles of *ZNF671* in Cancers

We next explored the functional roles of *ZNF671* in cancers, and analyzed the correlation between *ZNF671* expression and functional state. We found that *ZNF671* was positively associated with DNA damage ($R = 0.18$; $***P < 0.001$), apoptosis ($R = 0.13$; $*P < 0.05$), DNA repair ($R = 0.10$; $*P < 0.05$) in GBM; with stemness ($R = 0.11$; $*P < 0.05$) and inflammation ($R = 0.06$; $*P < 0.05$) in AST; with proliferation ($R = 0.29$; $**P < 0.01$), quiescence ($R = 0.23$; $*P < 0.05$), and differentiation ($R = 0.21$; $*P < 0.05$) in MEL; with inflammation ($R = 0.17$; $***P < 0.001$), metastasis ($R = 0.16$; $***P < 0.001$), stemness ($R = 0.15$;



** $P < 0.01$), hypoxia ($R = 0.13$; ** $P < 0.01$), EMT ($R = 0.13$; * $P < 0.05$), and differentiation ($R = 0.08$; * $P < 0.05$) in ODG; with EMT ($R = 0.12$; * $P < 0.05$) and hypoxia ($R = 0.12$; * $P < 0.05$) in glioma (brain); and with stemness ($R = 0.18$; * $P < 0.05$), and hypoxia ($R = 0.18$; * $P < 0.05$) in BRCA (Figures 3, 4).

However, *ZNF671* was negatively associated with DNA damage in ODG ($R = -0.11$; ** $P < 0.01$); with hypoxia ($R = -0.55$; * $P < 0.05$), EMT ($R = -0.50$; * $P < 0.05$), apoptosis ($R = -0.49$; * $P < 0.05$), angiogenesis ($R = -0.48$; * $P < 0.05$), and quiescence ($R = -0.43$; * $P < 0.05$) in glioma (PDX), and with inflammation ($R = -0.20$; * $P < 0.05$) and differentiation ($R = -0.18$; * $P < 0.05$) in BRCA (Figures 3, 4). These results indicated that *ZNF671* plays a different functional role in cancers and that the functional difference could be associated with the functional populations of cancer cells.

The Different Roles of *ZNF671* in Different Cell Groups

To determine the functionally heterogeneous roles of *ZNF671* in cancer cells, we inferred that single cells exhibited widespread heterogeneity in terms of their functional states in cancer. We applied t-SNE to reduce the non-linear dimensionality of the cancer cell data and placed different cell clusters on a t-SNE

map (Figure 5), which indicated that the cell groups might be associated with the functional heterogeneity of cancer.

To reveal the roles of *ZNF671* in different cell groups, we further explored functional roles and correlations of *ZNF671* in different cancer subgroups. As shown in Figure 6, *ZNF671* expression was positively associated with DNA repair, DNA damage, and apoptosis but negatively associated with angiogenesis, differentiation, and proliferation in MGH30 cell groups of GBM, while *ZNF671* expression was positively associated with proliferation in MGH31 cell groups of GBM. In glioma (brain), *ZNF671* expression was negatively correlated with angiogenesis in MUV1, with DNA repair, DNA damage, and cell cycle in MUV5, with DNA repair in BCH836, and with apoptosis in BCH869. *ZNF671* expression was positively correlated with hypoxia in MUV10, BCH836, and BCH869 in glioma (brain). In glioma (PDX), *ZNF671* expression in BCH869 correlated negatively not only with hypoxia but also with EMT, apoptosis, angiogenesis, and quiescence. In AST, *ZNF671* expression was positively correlated with stemness in MGH45 and MGH56, with invasion in MGH61, and with inflammation in MGH64, and it was negatively correlated with cell cycle and invasion in MGH45, with angiogenesis in MGH57, and with invasion in MGH64. In ODG, *ZNF671* expression was positively

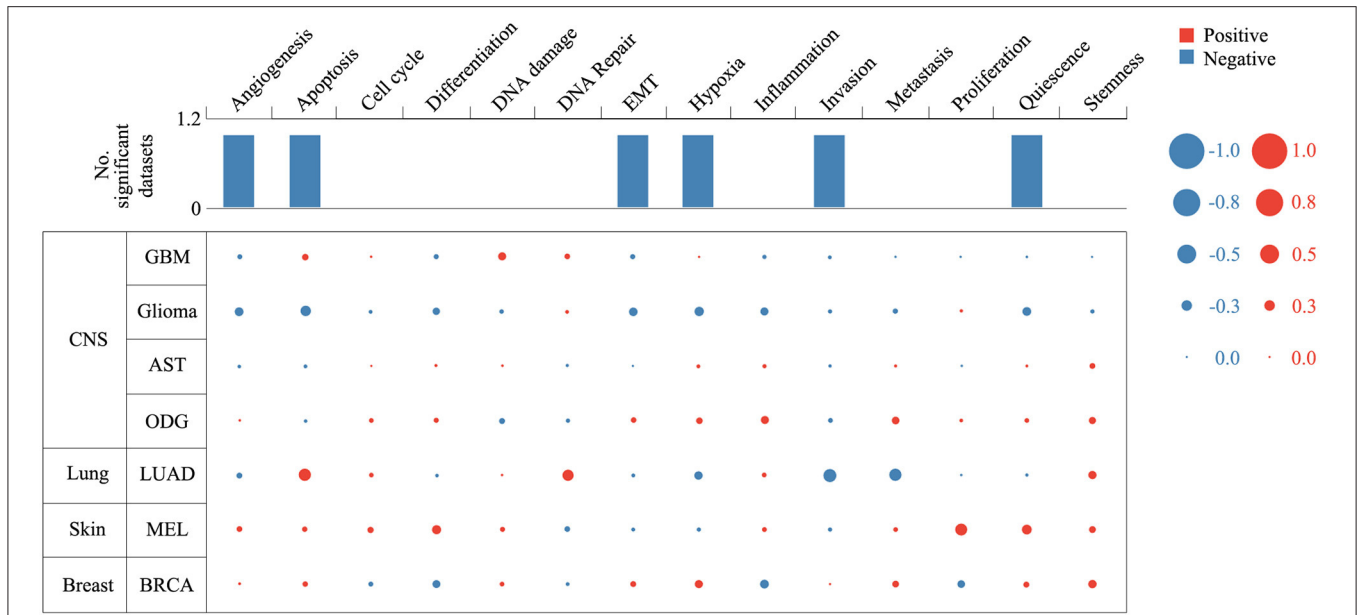


FIGURE 2 | Relevance of *ZNF671* across 14 functional states in distinct cancers. The upper bar chart shows the number of datasets in which *ZNF671* is significantly related to the corresponding state. In the bubble chart in the second section, a results table is used to display the basic information of all single-cell datasets in the selected cancer type and the corresponding correlations with the 14 functional states. Glioblastoma (GBM), Glioma (Brain), Glioma (PDX), Astrocytoma (AST), Oligodendrogloma (ODG), Lung adenocarcinoma (LUAD), Melanoma (MEL), and Breast cancer (BRCA).

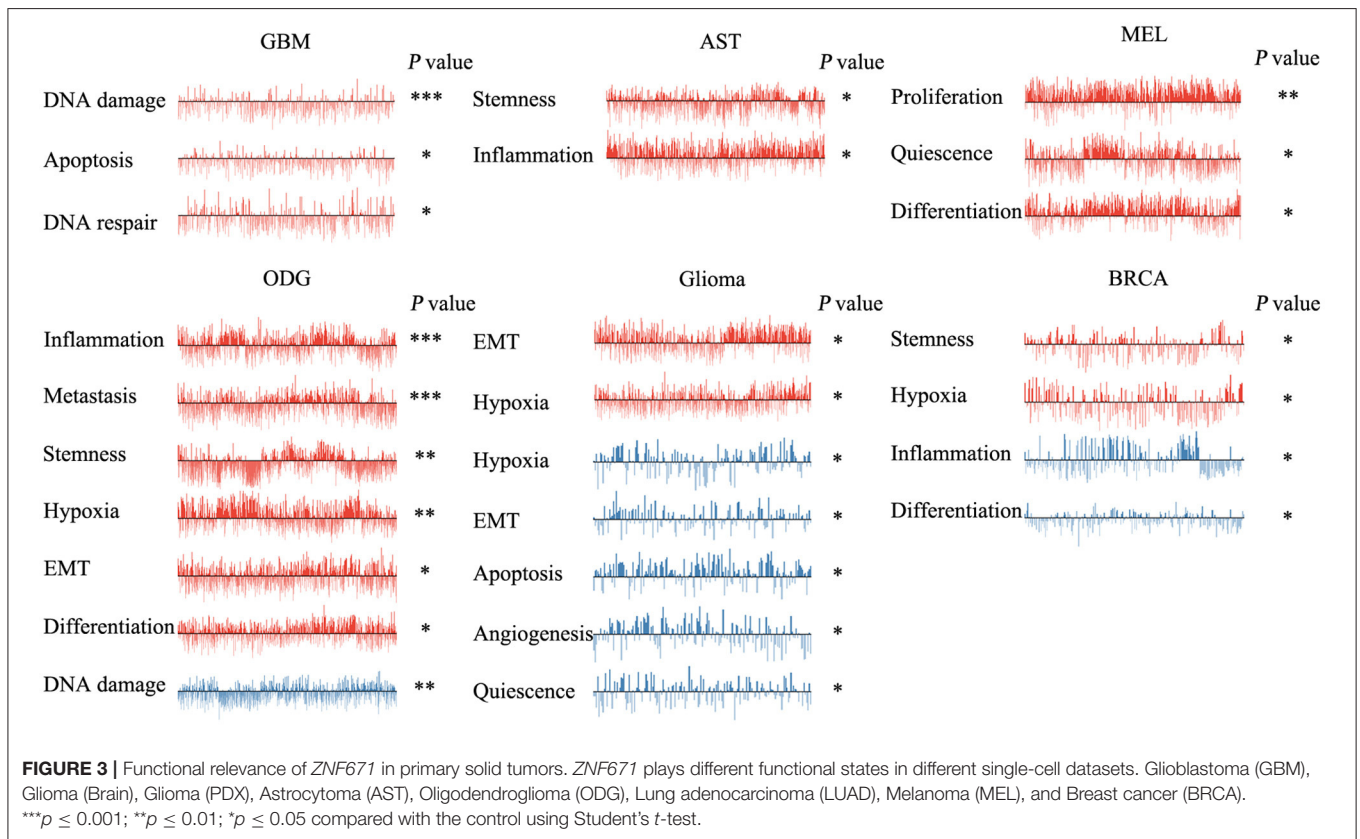


FIGURE 3 | Functional relevance of *ZNF671* in primary solid tumors. *ZNF671* plays different functional states in different single-cell datasets. Glioblastoma (GBM), Glioma (Brain), Glioma (PDX), Astrocytoma (AST), Oligodendrogloma (ODG), Lung adenocarcinoma (LUAD), Melanoma (MEL), and Breast cancer (BRCA). *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$ compared with the control using Student's *t*-test.

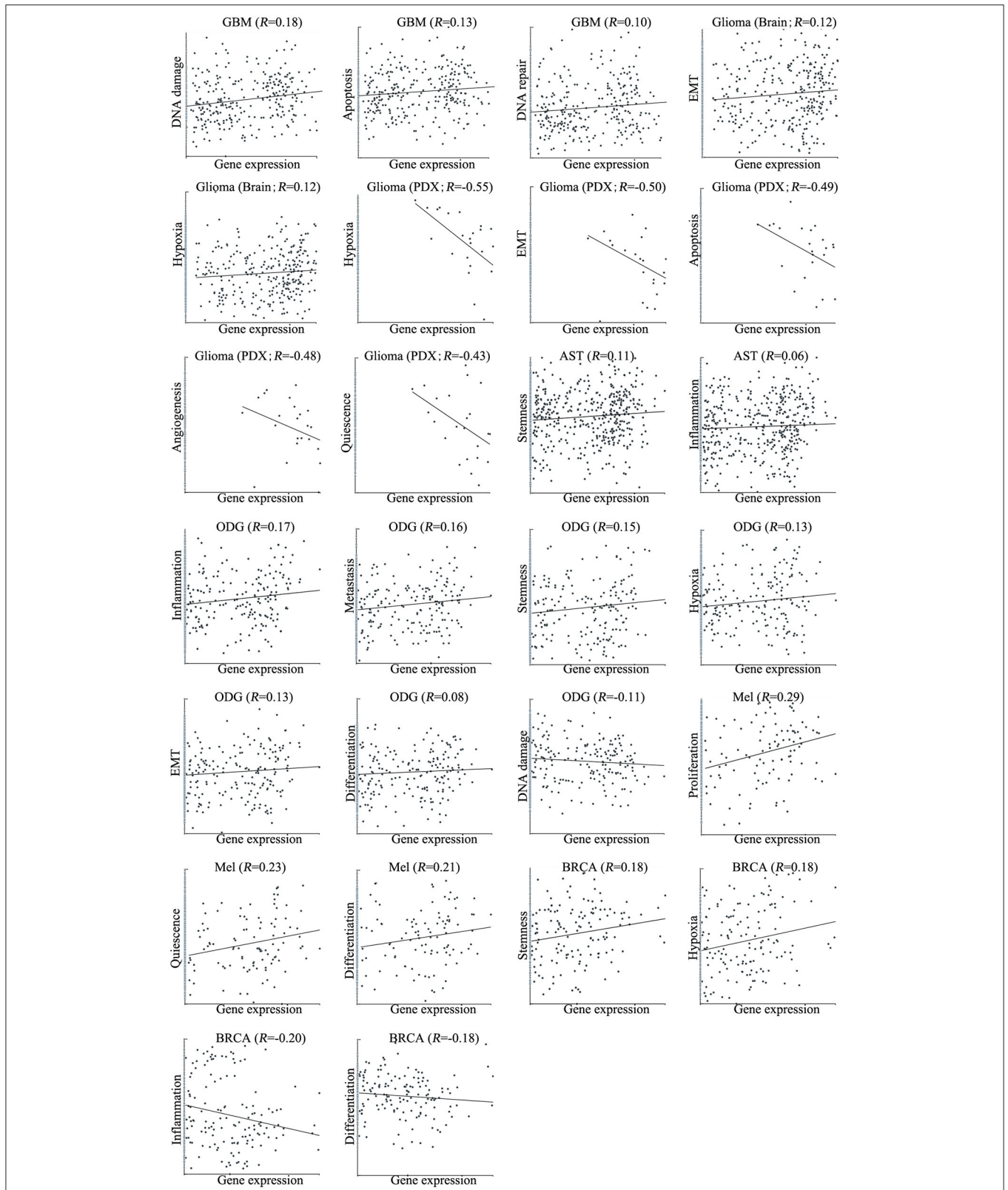
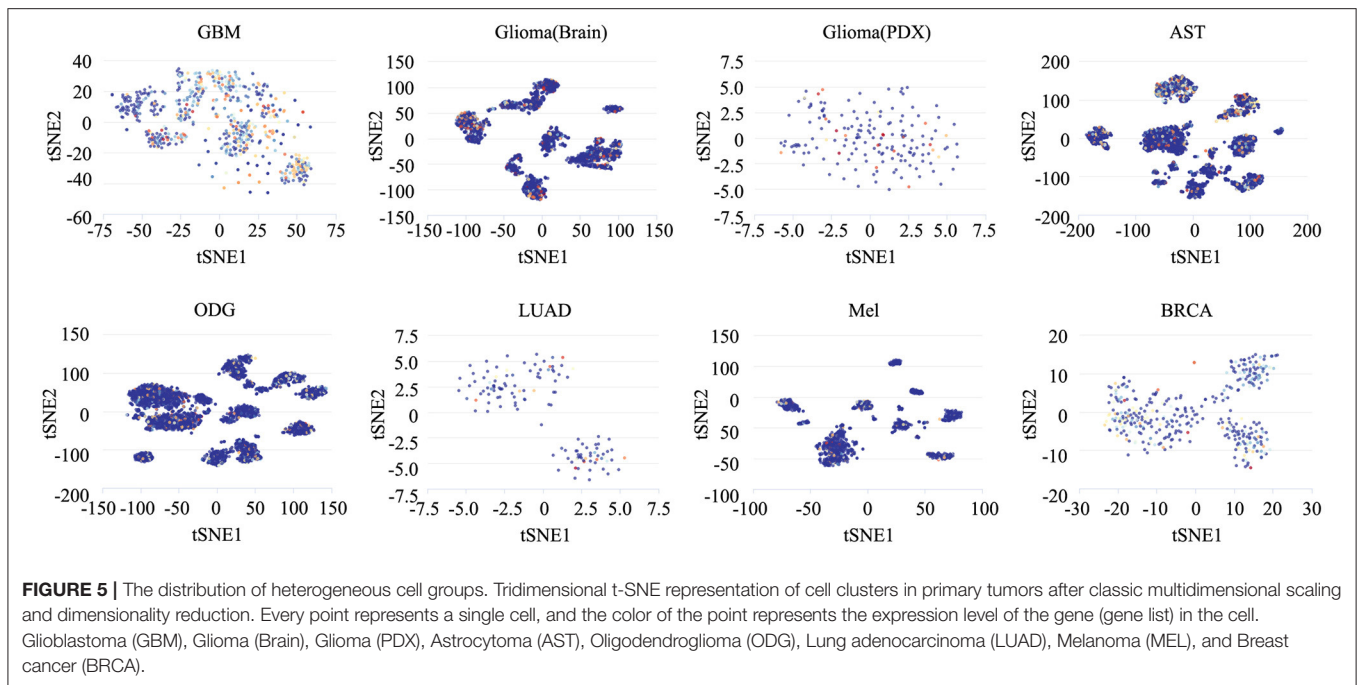


FIGURE 4 | Correlations between *ZNF671* mRNA expression and functional states in primary solid tumors. The detailed functional relevance in each specific cell group of a specific scRNA-seq dataset is shown. Glioblastoma (GBM), Glioma (Brain), Glioma (PDX), Astrocytoma (AST), Oligodendrogloma (ODG), Lung adenocarcinoma (LUAD), Melanoma (MEL), and Breast cancer (BRCA).



correlated with metastasis, hypoxia, inflammation, and apoptosis in MGH36 and with inflammation in MGH60 but negatively correlated with apoptosis in MGH54 and with quiescence in MGH93. Similarly, in MEL, *ZNF671* expression was positively correlated with stemness in tumor78, with proliferation and stemness in tumor79, with proliferation and differentiation in tumor88, and with inflammation in tumor89. However, *ZNF671* expression was negatively correlated with DNA repair in tumor78, DNA damage and angiogenesis in tumor80, and cell cycle in tumor89. In LUAD, *ZNF671* expression was positively correlated with DNA repair in MBT15 but negatively correlated with metastasis and invasion in PT45. In BRCA, *ZNF671* expression was only positively correlated with DNA damage in CSL KO xenograft tumor (Figure 6, all $*P < 0.05$; $**P < 0.01$).

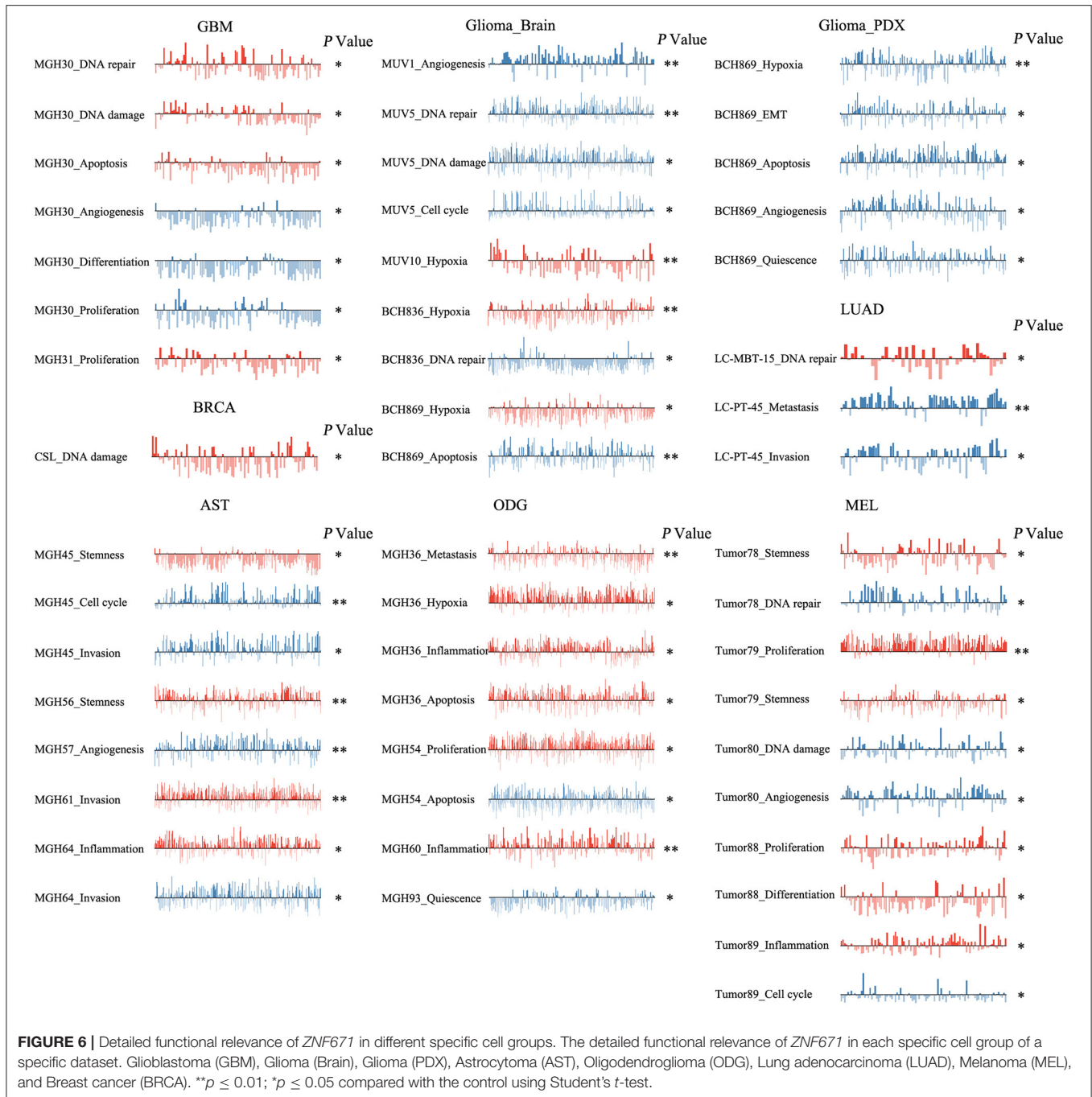
ZNF671* Inhibits Cell EMT, Migration, and Invasion *in vitro

To determine the functional roles of *ZNF671* in cancer cells, we performed Western blot assay and migration and invasion assays using U87, U251, A375, MDA-MB-231, and BT-549 cell lines transfected with *ZNF671* or vector plasmids. As shown in Figure 7A, Western blot analysis validated that *ZNF671* protein was obviously upregulated after transfection of *ZNF671* plasmid. Furthermore, the overexpression of *ZNF671* was associated with increased expression of the epithelial marker E-cadherin and decreased expression of the mesenchymal marker Vimentin. Transwell assays showed that overexpression of *ZNF671* inhibited cancer cell migration and invasion *in vitro* (Figures 7B–D). These findings indicate that *ZNF671* inhibits the EMT, migration, and invasion of U87, U251, A375, MDA-MB-231, and BT-549 cells *in vitro*.

DISCUSSION

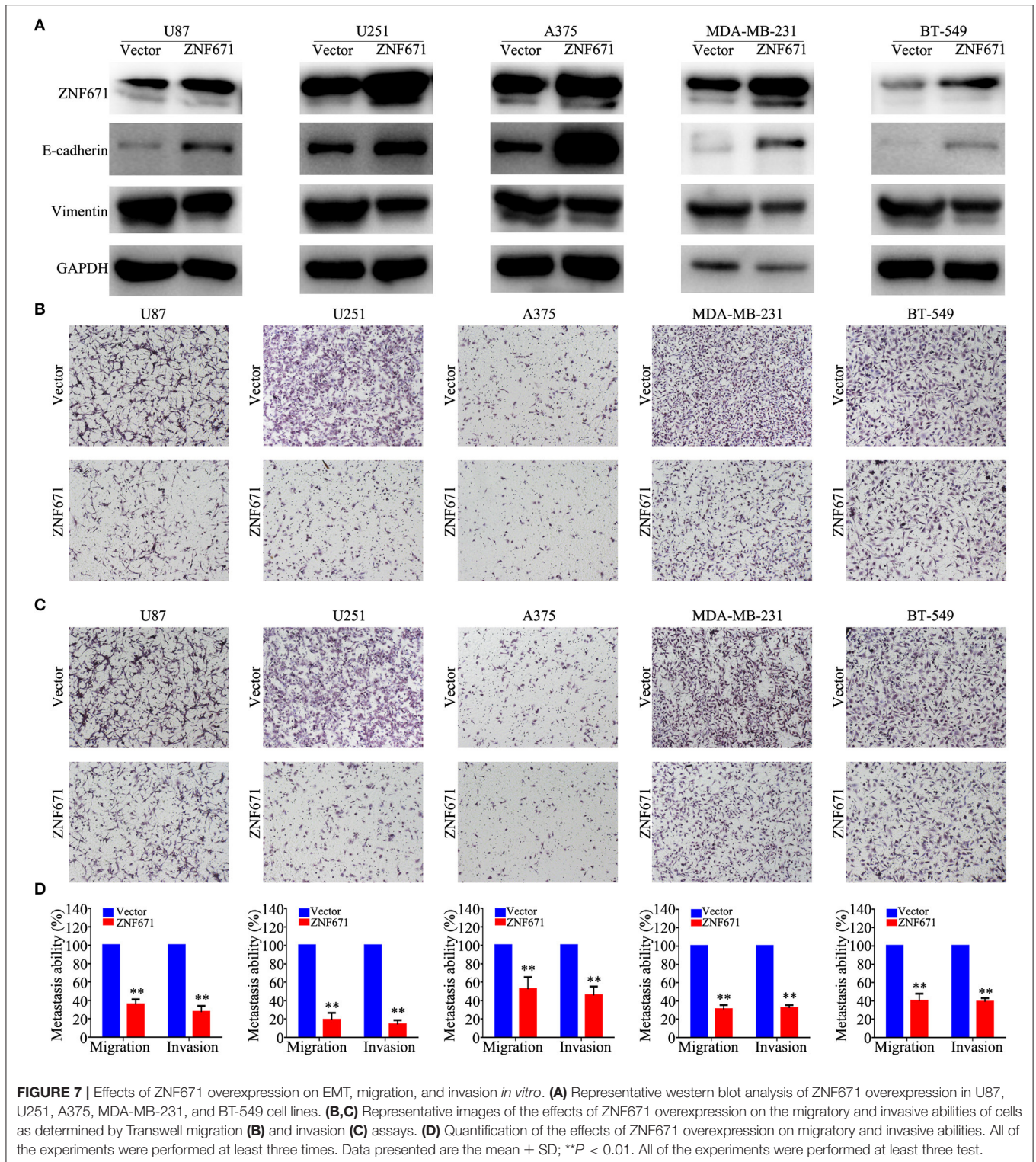
ZNF671, which contains C2H2-type zinc fingers (ZFs) and a Krüppel-associated box (KRAB) domain, is a member of the KRAB-ZF (KRAB-ZFP) transcriptional family. KRAB-ZFPs are involved in regulating angiogenesis (36), apoptosis (37–39), the cell cycle (40, 41), inflammation (42), invasion and metastasis (43, 44), and stemness (45). Our previous studies demonstrated that *ZNF671* is a tumor suppressor that is epigenetically silenced by DNA methylation in nasopharyngeal carcinoma, BRCA, CESC, HNSC, KIRP, LUAD, PAAD, and UCEC (26, 27). However, there is limited information regarding the role of *ZNF671* in cancer progression and development, and there have been no systematic studies of the role of *ZNF671* in cancer's heterogeneous functional states.

In this study, we found a total of eight solid tumor-related *ZNF671* scRNA-seq datasets, including GBM, glioma, AST, ODG, LUAD, MEL, and BRCA. ScRNA-seq functional state analysis showed that *ZNF671* played a tumor suppressor role and/or an oncogenic role in angiogenesis, apoptosis, cell cycle, differentiation, DNA damage, DNA repair, EMT, hypoxia, inflammation, invasion, metastasis, proliferation, quiescence, and stemness. The different functional states in tumors may be associated with the inherent heterogeneity of the tumor. However, the synthetic analysis of eight solid tumors showed that *ZNF671* was negatively associated with angiogenesis, apoptosis, EMT, hypoxia, invasion, and quiescence. Western blot and transwell assays showed that *ZNF671* inhibited EMT, migration, and invasion of CNS cancers, lung cancer, melanoma, and breast carcinoma *in vitro*. These results suggested a crucial tumor suppressor role for *ZNF671* in the progression of these cancers, which was consistent with our previous studies (26, 27).



To further explore the heterogeneous functional state of *ZNF671* in cancers, we applied t-SNE to describe the distribution of cells. We found different cell clusters on a t-SNE map and proposed that these cell subgroups might lead to cancer functional heterogeneity. Functional analysis of the cancer cell subgroups validated that the heterogeneous cell populations had different roles in cancer progression and development, which provided us with a fine level of resolution for cancer treatment. However, there were still several limitations. First, this study was based on current scRNA datasets, and

several scRNA datasets only contain data for hundreds of single cells, so more cells should be considered for analysis. Second, we found the *ZNF671* inhibits angiogenesis, apoptosis, EMT, hypoxia, invasion, and quiescence in CNS cancers, lung cancer, melanoma, and breast carcinoma. Moreover, we only identified that *ZNF671* suppresses cell EMT, migration, and invasion *in vitro*. The angiogenesis, apoptosis, hypoxia, and quiescence functional states need to be identified further, and the suppressor role of *ZNF671 in vivo* needs to be explored further.



In conclusion, this study systematically evaluated the tumor suppressor role of *ZNF671* based on scRNA-seq datasets. Our findings revealed that *ZNF671* is a tumor suppressor in LUAD, BRCA, GBM, glioma, AST, ODG, and MEL. However, the mechanism of *ZNF671*'s

tumor suppressor role remains unknown, and further studies are needed to clarify this issue. Our results provide new insights into the role of *ZNF671* in multiple tumors and identifies *ZNF671* as a novel target for cancer treatment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: <http://www.bioconductor.org>.

AUTHOR CONTRIBUTIONS

JZha, JLu, and HJ designed the research. TX, JZhe, YT, RL, BW, JLi, AX, and XH acquired and analyzed the data. JZha, HJ, and YY wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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